

Comparative analysis of the blood microbiome in Alzheimer's disease and healthy individuals

by

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Declaration

I, Diego Jose Castillo Vaca declare that the dissertation, which I hereby submit for the degree Magister Scientiae (M.Sc.) Genetics at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.



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“The mind that opens to a new idea never returns to its original size”

-Albert Einstein

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List of Abbreviations

AChEIs	Acetyl choline esterase inhibitors
AD	Alzheimer's disease
ANOVA	Analysis of variance
APOE	Apolipoprotein E
A β	Amyloid beta
bp	Base pairs
CVD	Cardiovascular disease
ddH ₂ O	Double distilled water
DFM	Dark-field microscopy
dl	Decilitre
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
<i>et al.</i>	<i>et alia</i>
Fe	Iron
FISH	Fluorescence <i>in situ</i> hybridization
fl	Femtolitre
GWAS	Genome wide association studies
Hb	Haemoglobin
ID	Identification
kb	Kilobases
kg	Kilogram

l	Liter
MCH	Mean corpuscular haemoglobin
MCV	Mean corpuscular volume
min	Minute/s
ml	Millilitre
ng	Nanograms
NGS	Next generation sequencing
NMDS	Non-metric multidimensional scaling
NTC	Negative control
OTU	Operetional taxonomic unit
PBMC	Peripheral blood mononuclear cells
PCoA	Principal coordinate analysis
PCR	Polymerase chain reaction
PERMANOVA	Permutational anlysis of variance
pg	Picograms
PMA	Propidium monoazide
QIIME	Quantitative Insights into Microbial Ecology
qPCR	Quantitative polymerase chain reaction
RBC	Red blood cell
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
TEM	Transmission electron microscopy

v/v	Volume per volume
V3	16S ribosomal ribonucleic (rRNA) acid gene hypervariable region 3
V4	16S ribosomal ribonucleic acid (rRNA) gene hypervariable region 3
w/v	Weight per volume
WBC	White blood cell
WMGS	Whole metagenome shotgun sequencing
α	Alpha
β	Beta
γ	Gamma
$^{\circ}\text{C}$	Degrees Celsius
μ	Micro
μl	Microlitre
μmol	Micromol

Summary

Human blood is no longer considered to be a sterile environment. Bacteria and other microorganisms may be present even in the blood of healthy individuals. Dysbiosis of the blood microbiome has been linked to different conditions such as diabetes, cirrhosis, liver- and cardiovascular disease. According to the Alzheimer's disease (AD) pathogen hypothesis, microorganisms might be involved in the origin and progression of this type of dementia. There seems to be a connection between the gut microbiome and this neurodegenerative disease, however a possible relationship between the blood microbiome and AD has not yet been investigated.

This dissertation aimed to investigate the blood microbiome in Alzheimer's patients and healthy individuals, with an emphasis on AD risk factors (sex, ABO blood group, and *APOE* status) and the blood environment (Fe serum level, white-, and red blood cells). The objectives of the study were to: 1) study the bacterial composition and diversity in the blood of AD patients and healthy individuals; 2) determine if there were any differences in the blood microbiome between AD patients, their co-habitants, and young and elderly healthy participants; 3) investigate whether AD risk factors and haematological parameters had an effect on the blood microbiome of Alzheimer's patients and healthy individuals. Next generation amplicon sequencing and qPCR of the 16S rRNA gene were performed, to determine microbial composition and bacterial load in the blood of all participants, respectively. Genotyping of the major risk gene for AD (*APOE*) was also achieved. Iron serum level, differential-, and total blood counts were measured in all participants, to investigate a possible link to haematological factors in the blood environment, where bacteria are potentially present.

As a result of the use of stringent negative controls in this study, it was possible to conclude that bacterial DNA is present in the blood of AD patients and healthy individuals. However, bacterial DNA concentration was not found to differ between the study groups, indicating that Alzheimer's patients do not present a higher or lower load of bacteria in their blood. Haematological parameters that forms part of the blood environment were gathered for all participants, and specific differences were observed in haemoglobin concentrations, red cell distribution width, lymphocytes and neutrophil counts, when the

different groups were compared with each other. Moreover, *APOE* genotype was successfully obtained for all participants, and a relationship of dependence was confirmed between the *APOE4* allele and presenting a positive AD diagnosis. The *APOE* genotype was not linked to bacterial taxonomy, alpha-, or beta diversity in the AD group. No differences in taxonomy were found between Alzheimer's patients and healthy participants. However, certain haematological parameters, such as red- and white blood cell counts, appeared to be linked to blood bacterial taxonomy in AD and healthy groups. Alpha diversity differed between Alzheimer's patients and healthy individuals, yet no differences in beta diversity were observed between different study groups. Alzheimer's patients presented a higher alpha diversity, which could be explained by the dysregulation of the immune system as a consequence of this disease. This was further supported by correlations found between specific white blood cells, such as monocytes and neutrophils, and bacterial alpha diversity. Future research needs to explore the relationship between the blood microbiome and the immune system.

Chapter 1

The human blood microbiome and Alzheimer's disease

1. Introduction

The human microbiome comprises a vast corpus of bacterial, archaeal, viral and fungal microbial taxa (LLOYD-PRICE *et al.* 2016). While most of these micro-organisms are commensal, many are mutualistic and some are pathogenic. Regardless of whether their presence is beneficial, inconsequential or detrimental, our lives are inextricably linked to the microbes with which we share our bodies. Bacteria comprise approximately 2% of the adult human body mass (1.5 kg), roughly equivalent in size to the human brain or liver (MOLINA AND DiMAIO 2012). Given our extensive co-evolutionary history with microbes (MOELLER *et al.* 2016), it is not surprising that the estimated number of unique bacterial genes in our 'accessory genome' (~3,300,000) exceeds the number of our own genes (~22,000) by a factor of 150 (QIN *et al.* 2010b). Human microbiome research, described as the study of the entire DNA content of micro-organisms inhabiting our bodies, has rapidly evolved over the past decade, and has been reviewed extensively (CHO AND BLASER 2012; KIM *et al.* 2013; MORGAN *et al.* 2013; KHANNA AND TOSH 2014; LLOYD-PRICE *et al.* 2016).

The exploration of our 'microbial-selves' has been facilitated largely by the introduction of Next Generation Sequencing (NGS) and the advent of whole metagenome shotgun sequencing (WMGS), as techniques to study microbial genetic material present in different human body-sites (SEGATA *et al.* 2013). For many years, scientists have aimed to establish a taxonomy-based set of core human-associated micro-organisms. However, a more valuable approach involves ascertaining the primary core microbial composition based on functional (metabolic) capacity, since it is easier to correlate pathogenesis with deviations or changes (*i.e.*, dysbiosis) in a 'core' microbiome (TURNBAUGH *et al.* 2009). In this regard, several large-scale population-based studies have sequenced the metagenomes of the human gut microbiome, as well as other medically-relevant body-sites including the skin, vagina and mouth. Two notable collaborative projects have been developed to achieve this fundamental aim. As part of the 'Metagenomes of the Human Intestinal Tract' project (QIN *et al.* 2010a; LE CHATELIER *et al.* 2013; LI *et al.* 2014) and the 'Human Microbiome Project' (HMP) (AAGAARD *et al.* 2013), more than 2,000 people from across the globe had contributed to the study of the microbiome structure of healthy

individuals since 2006 (LLOYD-PRICE *et al.* 2016). Although most contemporary research focuses on the human gut microbiome, the microbial communities present in the human mouth and eyes, on the skin, lungs and in the placenta and urogenital tracts have also been described (AAGAARD *et al.* 2013; BLEKHMANN *et al.* 2015; LLOYD-PRICE *et al.* 2016).

2. The blood microbiome

The prospect of the existence of a 'healthy' human blood microbiome has roused much interest in the scientific community (MCLAUGHLIN *et al.* 2002; BAHRANI-MOUGEOT *et al.* 2008; PAÏSSÉ *et al.* 2016). Human blood comprises ~54.3% plasma, ~45% red blood cells (erythrocytes), ~0.7% white blood cells (lymphocytes) and a variable number of platelets (thrombocytes), depending on health status (ALBERTS *et al.* 2002). Following the first documented observation of erythrocytes by Antonie van Leeuwenhoek in 1674 (BESSIS AND DELPECH 1981) (Fig. 1), it is now known that blood is the liquid medium that carries and sustains the most basic, but most essential, elements of life. Whereas erythrocytes are responsible primarily for the transport of oxygen, lymphocytes serve as a highly efficient surveillance system that monitors the blood for invasive microbes (JERNE 1973). The primary function of thrombocytes is to react to bleeding from blood vessel injury by clotting (BLACHE 1992). Because blood has traditionally been considered to be a sterile environment, devoid of all other forms of foreign (e.g., bacterial) cells, it is not surprising that the concept of a healthy HBM has been met with criticism (NIKKARI *et al.* 2001; MCLAUGHLIN *et al.* 2002; PAÏSSÉ *et al.* 2016).

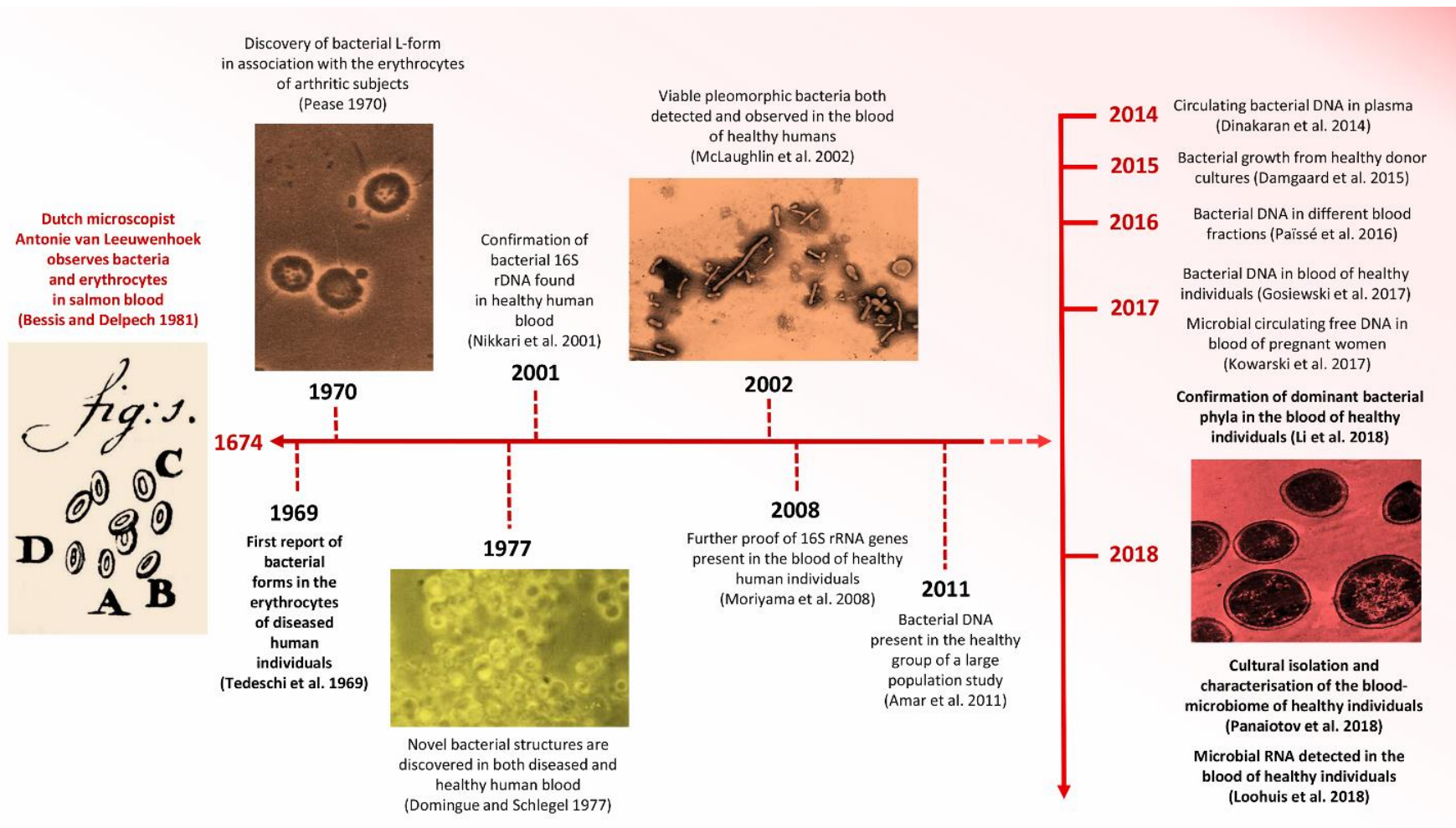


Fig. 1 Timeline indicating significant advances concerning the healthy human blood microbiome research. The timeline shows that more research have reported the existence of a human healthy blood microbiome in the past three years. Images modified from Bessis and Delpach (1981), Pease (1970), Domingue and Schlegel (1977), and Panaïotov *et al.* (2018).

The controversy concerning the incidence of foreign cells in human blood extends back to the late 1960s, when Guido Tedeschi and colleagues reported the presence of metabolically active bacteria in the blood of healthy human subjects (TEDESCHI *et al.* 1969) (Fig. 1). Specifically, the increased absorption of nucleosides and amino acids in erythrocyte suspensions led them to hypothesise that mycoplasm-like or L-phase (cell-wall deficient) bacteria were present in the blood of overtly non-diseased individuals. Nearly a decade later, in 1977, Gerald Domingue and Jorgen Schlegel reported that ~7% of blood samples derived from a healthy human cohort exhibited bacterial growth following osmotic lysis and filtering (DOMINGUE AND SCHLEGEL 1977).

More recent evidence for the hypothetical existence of a healthy HBM derives from Nikkari and colleagues (2001), who reported the presence of bacterial DNA in the blood of a healthy human cohort. This study, based on qPCR, included the use of rRNA-specific fluorescent probes and 16S rRNA-specific primers and identified bacterial taxa belonging to five divisions and seven phylogenetic groups (NIKKARI *et al.* 2001). This study is however limited by the fact that all observations are based on the analysis of the blood of only four individuals. Shortly thereafter, McLaughlin and colleagues (2002) described the presence of pleomorphic bacteria in the blood of individuals who did not present any perceptible clinical manifestations of disease. In this study, transmission electron microscopy (TEM), dark-field microscopy (DFM), fluorescence *in situ* hybridisation (FISH) and the sequencing of PCR-amplified 16S rRNA and *gyrB* genes confirmed the presence of bacterial DNA in the blood of healthy individuals (MCLAUGHLIN *et al.* 2002). Correspondingly, Moriyama and colleagues (2008) contributed to the concept of a 'healthy' HBM by confirming the presence of bacterial 16S rRNA genes in the blood of healthy humans (MORIYAMA *et al.* 2008).

As anticipated, challenging the traditional conviction concerning the sterility of blood in healthy humans under normal circumstances has generated considerable controversy. Mitchel and colleagues (2016) assessed the findings of McLaughlin and colleagues (2002) and other studies, concluding that the pleomorphic bacteria identified in the blood of healthy humans were, in fact, nothing more than "micro-particles" derived from disintegrated erythrocytes (MITCHELL *et al.* 2016). Martel and colleagues (2017) supported

this argument with the discovery that bacteria-like structures closely resembled membrane vesicles and that vibrating refringent particles captured by dark-field microscopy were merely aggregates of blood proteins (MARTEL *et al.* 2017). Although the visual confirmation of micro-organisms present in the blood of healthy individuals requires further examination, evidence confirming the presence of microbial genetic material in the blood-circulatory system is accumulating (McLAUGHLIN *et al.* 2002; MORIYAMA *et al.* 2008; PAÏSSÉ *et al.* 2016; LOOHUIS *et al.* 2018).

The application of innovative analytical technologies, such as targeted NGS of the 16S rRNA gene as well as WMGS, has provided increasingly robust evidence for a non-diseased human blood microbiome (AMAR *et al.* 2011; AMAR *et al.* 2013; DECUYPERE *et al.* 2016; PAÏSSÉ *et al.* 2016; LI *et al.* 2018). RNA-sequence data has also contributed to this premise, as bacterial transcripts have been identified in non-diseased control groups (LOOHUIS *et al.* 2018). Researchers characterising the blood-microbiome in diseased patients have also detected genetic material in their healthy control groups (Table 1). Moreover, the presence of comparable bacterial phyla appears to lend support for the existence of a healthy human blood microbiome (McLAUGHLIN *et al.* 2002; AMAR *et al.* 2011; DAMGAARD *et al.* 2015; GOSIEWSKI *et al.* 2016; PAÏSSÉ *et al.* 2016; KOWARSKY *et al.* 2017; LOOHUIS *et al.* 2018).

Table 1: Studies investigating the human blood microbiome and the detection of bacteria in the blood of healthy cohorts.

Study population	Healthy microbiome indications	Method of detection	Major findings	Reference
One-hundred male and female subjects selected at random.	Study consisted of healthy individuals.	Radioactive uptake of nucleosides and amino acids in erythrocyte suspensions.	Possible presence of mycoplasm-like or L-phase bacterial forms in the bloodstream of overtly healthy individuals.	Tedeschi <i>et al.</i> (1969)
Ninety-five diseased patients and 60 healthy individuals.	Bacterial growth observed in 7% of the healthy cohort.	Filtrated blood used to culture bacteria.	Novel bacterial structures seen in blood suggesting bacterial phase before becoming ordinary bacteria.	Dominique & Schlegel (1977)
Four individuals without clinical signs of disease.	Study population consisted of healthy individuals only.	qPCR including the use of rRNA gene-specific fluorescent probes.	Presence of bacteria from five divisions and 7 distinct phylogenetic groups in the blood of healthy individuals.	Nikkari <i>et al.</i> (2001)

Table 1 continuation

Twenty-five healthy individuals.	Study population consisted of healthy individuals only.	PCR amplification of 16S rRNA and <i>gyrB</i> genes. Used microscopy (dark-field and TEM) and fluorescent <i>in situ</i> hybridization.	Confirms the existence of pleomorphic bacteria in 'healthy' blood. Bacteria exhibited limited growth and susceptibility to antibiotics. Sequencing revealed the presence of <i>Proteobacteria</i> (possibly <i>Pseudomonas</i>).	McLaughlin <i>et al.</i> (2002)
Two healthy individuals.	Study population consisted of healthy individuals only.	PCR of 16S rRNA gene and Sanger sequencing of different clones.	16S rRNA genes in 'healthy' blood confirmed. Bacteria identified only in clones (<i>Aquabacterium</i> , <i>Budvicia</i> , <i>Stenotrophomonas</i> , <i>Serratia</i> , <i>Bacillus</i> and <i>Flavobacteria</i>).	Moriyama <i>et al.</i> (2008)
Comprising 3,280 patients from the DESIR study.	Healthy (non-diabetic) patients also presented bacterial DNA in their blood.	Quantification of 16S rRNA gene with broad-range quantification kit and pyro-sequencing.	16S rRNA gene concentration higher in individuals that developed diabetes. A core blood microbiome, mostly consisting of <i>Proteobacteria</i> , found in all study groups.	Amar <i>et al.</i> (2011)
Comprising 3,936 patients from the DESIR study.	Bacterial DNA in blood of individuals not presenting CVD.	16S rRNA gene qPCR.	<i>Eubacteria</i> and <i>Proteobacteria</i> identified in groups affected by and free of cardiovascular disease.	Amar <i>et al.</i> (2013)
Eighty CVD patients and 40 healthy individuals.	Healthy control group presented circulating bacterial DNA in their plasma.	16S rRNA and β globin qPCR and shotgun sequencing of circulating DNA from blood plasma.	Bacterial DNA and microbial diversity higher in CVD group. <i>Proteobacteria</i> and <i>Actinobacteria</i> dominant in CVD group (<i>Actinobacteria</i> and <i>Proteobacteria</i> dominant in control group).	Dinakaran <i>et al.</i> (2014)
Fifty type-2 diabetes patients and 50 control individuals.	Bacterial rRNA detected in 4% of healthy individuals (opposed to 28% in diabetes patients).	16S rRNA RT-qPCR.	Higher detection rate of potential gut bacteria in the blood of patients with type-2 diabetes than in control group.	Sato <i>et al.</i> (2014)
Sixty self-reported healthy individuals older than 49 years.	Bacterial growth observed in 62% of healthy individuals.	Blood suspensions incubated on trypticase soy blood agar (TSA) or blue lactose plates, and identified by 16S rRNA colony PCR.	Bacterial growth observed in 35% of RBC fractions and 53% of plasma fractions. <i>Staphylococci</i> , <i>Propionibacterium</i> , <i>Micrococcus</i> and <i>Bacillus</i> most frequently found.	Damgaard <i>et al.</i> (2015)
Thirty healthy blood donors (18 to 53 years old).	Study population consisted of healthy individuals only.	16S rRNA gene qPCR and 16S targeted metagenomic sequencing (Illumina MiSeq).	Bacterial DNA present in buffy coat, erythrocytes and plasma. Most bacterial DNA corresponds to <i>Proteobacteria</i> and <i>Actinobacteria</i> (<i>Firmicutes</i> and <i>Bacteroidetes</i> also found).	Païssé <i>et al.</i> (2016)

Table 1 continuation

Twenty-three healthy individuals and 62 patients with sepsis.	Bacterial DNA found in all samples including 23 healthy individuals.	16S rRNA targeted metagenomic NGS (Illumina MiSeq).	Healthy samples presented higher diversity than sepsis patients. Abundance of <i>Proteobacteria</i> decreased in healthy individuals, while <i>Actinobacteria</i> decreased in sepsis group.	Gosiewski <i>et al.</i> (2016)
Nine cirrhosis patients and 9 healthy individuals (~60 years of age).	Bacteria found in 2 out of the 9 control individuals.	Microbial DNA qPCR (16S rRNA target gene).	Number of bacterial species and amount of bacterial DNA increased in cirrhotic patients.	Traykova <i>et al.</i> (2017)
Twenty-one bone marrow transplant-, 59 lung transplant- and 76 heart-transplant patients (32 pregnant participants).	Sequencing reads obtained from all transplant patients and pregnant participants.	Shotgun sequencing of cell-free DNA. Confirmation of novel contigs by direct PCR.	Circulating free-DNA from novel uncharacterized bacteria and viruses that could be members of the human gut microbiome found in the blood of all participants.	Kowarsky <i>et al.</i> (2017)
Fifty patients with severe acute pancreatitis and 12 healthy individuals.	Bacterial DNA found in all healthy participants.	16S rDNA gene qPCR and targeted metagenomic sequencing using Ion Torrent.	Higher number of 16S rDNA gene copies in patients. Healthy phyla include <i>Proteobacteria</i> , <i>Actinobacteria</i> , <i>Firmicutes</i> and <i>Bacteroidetes</i> . Increase of <i>Bacteroidetes</i> and a decrease of <i>Actinobacteria</i> observed in patients.	Li <i>et al.</i> (2018)
Twenty-eight blood samples from healthy individuals.	All blood samples were culture positive (confirmed by gram staining and TEM).	16S rRNA genes and ITS2 targeted sequencing on Illumina MiSeq and TEM.	Cultural isolation and characterisation of the blood microbiome of healthy individuals.	Panaiotov <i>et al.</i> (2018)
Comprising 192 individuals (48 schizophrenic, 47 diagnosed with lateral sclerosis, 48 with bipolar disorder and 49 healthy).	Bacterial transcripts identified in blood samples from healthy individuals.	High throughput RNA sequencing.	Prevalent phyla across study groups were <i>Proteobacteria</i> , <i>Firmicutes</i> and <i>Cyanobacteria</i> . Microbial diversity in schizophrenia patients significantly increased.	Loohuis <i>et al.</i> (2018)

In addition to challenging the *status quo* of the ‘germ-free’ human blood paradigm, methodological obstacles have hindered human blood microbiome research. Many micro-organisms found naturally within human blood may in fact be in a dormant or ‘suspended’ state (POTGIETER *et al.* 2015). Accordingly, culture-based methods cannot be reliably employed to support the existence of a blood microbiome. Furthermore, while the

concentration of bacterial DNA in the blood is typically very low, increasingly-sensitive analytical techniques, particularly qPCR, targeted NGS and WMGS may substantiate current evidence for the presence of 'innocuous' bacterial taxa in the blood of healthy humans (PAÏSSÉ *et al.* 2016).

Rigorous experimental controls, which are essential when studying low-biomass microbiomes prone to contamination from external sources, are not always included. This is particularly problematic as the detection of >90 microbial genera in DNA extraction and library preparation controls (SALTER *et al.* 2014; LAUDER *et al.* 2016) highlights the influence that contaminants derived from reagents and laboratory environments exert on sequence-based human microbiome analyses. The analyses of negative DNA extraction controls, as performed by Moriyama and colleagues (2008), indicated significantly-less 16S rRNA gene amplification when compared to blood derived from healthy individuals. These controls comprised saline water which had been in contact with povidone iodine sterilised skin (MORIYAMA *et al.* 2008). Conversely, the analyses of comparable samples as negative controls for 16S rRNA targeted Illumina MiSeq WMGS indicated virtually no amplified 'contaminant' taxa (DINAKARAN *et al.* 2014). Although a number of these samples did yield >10,000 DNA sequence reads, their taxonomic composition differed significantly from that of both the diseased and healthy blood samples. While investigating the blood-microbiome of cirrhotic patients, Traykova and colleagues (2017) recovered bacterial DNA from the blood of >20% of the healthy cohort. In this study, sterile water and pan-bacterial assays, which detect a broad range of bacterial taxa, were used as negative and positive controls (TRAYKOVA *et al.* 2017). The use of controls was also implemented when the blood-microbiome in different blood fractions was characterised (PAÏSSÉ *et al.* 2016). In a recent study by Loohuis and colleagues (2018) the importance of including stringent controls when studying low biomass microbiomes, such as human blood, was clearly demonstrated. While investigating the blood-microbial transcriptomes of both healthy human individuals and of patients affected with brain disorders, RNA obtained from lymphoblast cell lines were used as negative controls, and cells infected with *Chlamydia*, as positive controls. RNA reads were identified only for the *Chlamydiae* phylum in the positive controls, and no microbial sequences were detected in the lymphoblast cells (LOOHUIS *et al.* 2018).

It is evident that further research is required to establish whether the microbial DNA and RNA found in healthy human blood represents either living or dead, or active or non-active bacterial taxa. Although contamination derived from human sources poses a significant challenge to blood-microbiome research, bacteriological activity in the blood could potentially be studied through viability assay techniques such as propidium monoazide (PMA) treatment and cellular energy measurements (EMERSON *et al.* 2017). However, there is presently no specific and reliable means of detecting living bacteria in human blood.

Despite the fact that evidence for the presence of bacterial taxa comprising a healthy blood-microbiome in humans is accumulating, not much is known about the presence of other micro-organisms, such as viruses, Archaea and lower eukaryotes (*i.e.* fungi) in the blood of healthy humans. The presence of archaeal DNA is generally not reported (NIKKARI *et al.* 2001; MCCLAUGHLIN *et al.* 2002; MORIYAMA *et al.* 2008; DINAKARAN *et al.* 2014; DAMGAARD *et al.* 2015; GOSIEWSKI *et al.* 2016; PAÏSSÉ *et al.* 2016), presumably due to the typically low abundance or the absence of Archaea from blood samples. Dinakaran and colleagues (2014) did however document a relative abundance of 0.01% circulating archaeal DNA in the blood plasma of healthy individuals. The fungal microbiomes of the human intestinal tract, mouth, skin, lungs, and other body-sites have been explored (CUI *et al.* 2013; HUFFNAGLE AND NOVERR 2013). As the presence of fungi in the blood of healthy individuals has only recently been reported (PANAIOTOV *et al.* 2018), more research concerning the human blood myco-biome, in particular studies including stringent negative controls, is required. Regarding a tentative human blood-virome, and following the exclusion of taxa attributable to contamination, Moustafa and colleagues (2017) recovered 19 viral taxa from 42% of overtly healthy individuals. Previous reports have confirmed the presence of eukaryotic viruses, such as rhabdoviruses (STREMLAU *et al.* 2015), anelloviruses (FURUTA *et al.* 2015), and other families including *Herpesviridae* and *Poxviridae* (RASCOVAN *et al.* 2016) in healthy human blood. Additional research concerning the human blood-virome is therefore required to determine whether viruses are resident members of the HBM, or simply remnants of previous infections.

2.1. Location of the blood microbiome

With regards to the precise location of micro-organisms inside human blood, current evidence suggest that bacterial taxa may survive inside both erythrocytes and leukocytes. *Chlamydia pneumoniae*, an obligate intracellular bacterium and the major causative agent of pneumonia, has been found to inhabit peripheral blood mononuclear cells (PBMCs) in healthy individuals (YAMAGUCHI *et al.* 2004). Other bacteria, for example *Staphylococcus aureus*, can also invade and persist in white blood cells (WBCs). As far back as 2000, Gresham and colleagues showed that these bacteria both reside and retain their virulence within neutrophils (GRESHAM *et al.* 2000). Thwaites and Gant (2011) has also suggested that WBCs, and especially neutrophils, could act as ‘Trojan horses’ by offering protection against human antibodies, thereby facilitating the dissemination of *S. aureus* to different body-sites. Moreover, when Païssé and colleagues (2016) analysed the blood-microbiome of healthy individuals, most bacterial DNA (93.74 %) was found to be localised within the buffy coat (BC), which consists primarily of WBCs and platelets. A correlation between leukocyte concentration and the number of 16S rRNA gene copies in the BC of study participants was also identified. Similarly, some bacteria can enter RBCs directly, and persist within the nutrient-rich environment; it has been shown that *S. aureus*, a species commonly found in both healthy and diseased human gut microbiome (GRICE *et al.* 2009), can utilise iron (Fe) present in RBCs as a nutrient source (YAMAGUCHI *et al.* 2013). Yamaguchi and colleagues (2013) also showed that *Streptococcus pneumoniae*, a bacterium implicated in the onset of pneumonia and sepsis, became increasingly viable when incubated with erythrocytes. Similarly, it has been reported that *Brucella melitensis*, the causative agent of ovine brucellosis, and *Francisella tularensis*, a Gram-negative bacterium that causes tularaemia, also possess the capacity to invade and persist in erythrocytes (HORZEMPA *et al.* 2011; VITRY *et al.* 2014; A KOHLER *et al.* 2016).

2.2. Composition of the human blood microbiome

Despite the fact that the existence of a blood-microbiome in healthy human individuals appears to be supported by recent studies (GOSIEWSKI *et al.* 2016; PAÏSSÉ *et al.* 2016; KOWARSKY *et al.* 2017; TRAYKOVA *et al.* 2017; LI *et al.* 2018, LOOHUIS *et al.* 2018),

knowledge of the phylogenetic diversity of blood-borne bacteria remains limited. In contrast to the dominant bacterial phyla typically observed in the gut (*i.e.*, *Firmicutes* and *Bacteroidetes*), the human blood microbiome appears to be dominated by the phyla Proteobacteria and Actinobacteria (McLAUGHLIN et al. 2002; AMAR et al. 2011; DINAKARAN et al. 2014; DAMGAARD et al. 2015; GOSIEWSKI et al. 2016; PAÏSSÉ et al. 2016; KOWARSKY et al. 2017; LOOHUIS et al. 2018). The characterisation of blood bacterial diversity, however, varies between studies. In 2008, Moriyama and colleagues identified a set of bacterial taxa in their study of bacteria from the blood of two healthy individuals comprising mostly *Bacillus*, *Flavobacteria*, *Stenotrophomonas* and *Serratia*. Using a culture-based approach, Damgaard and colleagues (2015) observed bacterial growth in the blood of ~62% of healthy individuals. The most prominent taxa detected were *Propionibacterium acnes* and *Staphylococcus epidermis*, as well as *Bacilli* and *Micrococcus* species. In 2016, Païssé and colleagues analysed bacterial DNA present in different fractions of human blood. At class level, Fusobacteria and Flavobacteria were more abundant in RBCs, while members of the *Clostridia* class were dominant in plasma and erythrocyte fractions. Seven genera were identified in the RBC fraction, including two opportunistic pathogens, namely *Acinetobacter baumannii* and *Stenotrophomonas maltophilia* (PAÏSSÉ et al. 2016). Problematically, although the incidence of diverse blood taxonomic composition might indeed reflect actual microbial configuration, bacterial DNA found to contaminate DNA extraction kits typically includes *Bacillus*, *Flavobacteria*, *Fusobacteria*, *Propionibacterium* and *Serratia* (GLASSING et al. 2016). In addition to a critical awareness of potentially contaminating taxa, there is also a need for much broader metagenomic studies encompassing larger cohorts of both healthy and diseased individuals, as these would provide valuable insights into the composition of the putatively 'healthy' human blood microbiome, as well as its functionality and potential role in maintaining optimal human health and the onset of disease.

2.3. Clinical relevance

With reference to the role of the human microbiome in pathogenesis, the concept of 'dysbiosis', which refers to a change in the composition of symbiotic or commensal microbial communities (PETERSEN AND ROUND 2014), is particularly relevant. Although it

is not known whether dysbiosis is a cause, or simply a reflection, of a diseased state (BÄCKHED *et al.* 2012), numerous studies have related changes in human microbial community composition with the pathology of the disease the onset of disease. Examples include diabetes (QIN *et al.* 2012), asthma (TEO *et al.* 2015), inflammatory bowel disease (MORGAN *et al.* 2012), autism (PARRACHO *et al.* 2005) and even complex disorders such as Alzheimer's disease (AD) (PISTOLLATO *et al.* 2016). While considerable research has been dedicated to address the relationship between the gut microbiome and human health, a limited number of studies have explored dysbiosis of the human blood microbiome and its potential role in pathogenesis. Conditions such as diabetes, pancreatitis and also cardiovascular- and liver-disease have, however, been related to changes in the blood microbiome. Using a gut microbial qPCR microarray, the screening for bacterial DNA in the blood of both cirrhotic and healthy individuals by Traykova *et al.* (2017) resulted in the detection of higher levels of bacterial diversity in patients with cirrhosis, as opposed to a healthy control cohort. They also reported an increase of total bacterial DNA concentration in the blood of the diseased cohort compared to the healthy controls (TRAYKOVA *et al.* 2017). The human blood microbiome in patients with severe acute pancreatitis has also been analysed by 16S rRNA amplicon sequencing (KOWARSKY *et al.* 2017). Microbial taxonomic diversity was found to be reduced when compared to the healthy human cohort, and an increase in *Bacteroidetes* and a decrease in *Actinobacteria* were observed in pancreatitis patients. At class level, Bacteroidia and Clostridia increased in abundance, while Actinobacteria, Flavobacteria and Bacilli were reduced in the diseased group when compared to healthy controls (KOWARSKY *et al.* 2017). These variations in dominant taxa are strongly suggestive of blood-microbiome dysbiosis in pancreatitis patients. The onset of cardiovascular diseases may also be linked to blood microbiome dysbiosis. In 2013, Amar and colleagues discovered that the blood of patients who presented an acute cardiovascular event, even years following sample collection, had a significant decrease of total bacterial DNA when compared to a healthy cohort, as well as an increase in taxa assigned to the Proteobacteria. Accordingly, it was concluded that dysbiosis in the human blood microbiome could serve as a 'marker' for cardiovascular disease (CVD) prediction (AMAR *et al.* 2013). One year later, Dinakaran *et al.* (2014) also proposed the likelihood of an increase in microbial diversity and bacterial

DNA concentration when analysing cell-free DNA circulating in the blood of CVD patients. In this study, it was observed that Actinobacteria were dominant over Proteobacteria in CVD patients, while an opposite trend was observed in the healthy cohort (DINAKARAN *et al.* 2014).

The association between human blood microbiome dysbiosis and the onset of liver-disease has also been explored (LELOUVIER *et al.* 2016; SCHIERWAGEN *et al.* 2018), resulting in the proposal that blood-microbiota could serve as biomarkers for non-alcoholic fatty liver disease (NAFLD) prediction in obese patients (LELOUVIER *et al.* 2016). In this study, qPCR and 16S rRNA targeted metagenomic sequencing was employed. Liver fibrosis patients exhibited higher concentrations of 16S rRNA in their blood when compared to non-diseased participants. In addition, unique bacterial taxonomic clustering was observed in patients suffering from severe liver fibrosis (LELOUVIER *et al.* 2016). In 2018, Schierwagen and colleagues performed 16S rRNA NGS analyses on blood samples obtained from the portal vein, central and peripheral venous blood and liver outflow in patients suffering from liver fibrosis. Their findings corroborated those in the NAFLD study (LELOUVIER *et al.* 2016). Furthermore, each of the above mentioned circulatory compartments exhibited a unique taxonomic composition at genus level (SCHIERWAGEN *et al.* 2018).

In addition to these examples, various other studies have established possible associations between blood-derived bacteria originating from the gut microbiome, and the onset of diabetes; whereas gut bacteria have been detected to occur in ~28% of diabetes patients, healthy participants exhibited only ~4% gut microbiome-derived bacterial taxa (SATO *et al.* 2014). The most abundant taxa identified in the diabetes group included *Clostridium coccoides* and the *Atopobium* cluster (SATO *et al.* 2014). Although Amar and colleagues (2011) could not convincingly demonstrate a significantly different blood microbiome in patients prone to the development of diabetes, they did observe a higher 16S rRNA gene concentration in the blood of participants. Consequently, high concentrations of blood-derived bacterial DNA could potentially be used as a predictive marker for this condition (AMAR *et al.* 2011).

3. Alzheimer's disease

In 1906 Alois Alzheimer, a German psychiatrist, reported on a 50-year old woman who presented a peculiar condition affecting her cerebral cortex (BERRIOS 1990). Symptoms included severe loss of memory and cognitive ability, psychosocial impairment, and other features affecting her higher cortical functions. After her death, he found neurofibrillary tangles and senile plaques in her brain (BERRIOS 1990; SELKOE 2001). The original early-onset rare dementia described in those first years is now indistinct from its more common form, which largely affects the elderly, usually above the age of 65 years old (SELKOE 2001). It is estimated that AD currently affects more than 40 million people worldwide (SELKOE AND HARDY 2016).

3.1. *AD and amyloid beta*

Alzheimer's dementia has been part of our history for more than a century, however, the exact pathogenesis and pathophysiology of the disease remains to be unravelled. The amyloid hypothesis has been widely accepted for more than 30 years. (SELKOE AND HARDY 2016). Glenner and Wong (1984) purified and studied a protein obtained from fibrils in patients affected by cerebral amyloidosis. They proposed that this protein could be further investigated to gain more insights into the pathogenesis of the disease, and potentially be used to diagnose AD (GLENNER AND WONG 1984). Since this discovery, the amyloid β ($A\beta$) peptide, found in the characteristic senile plaques in Alzheimer's patients' brains, has been one of the main focuses of AD research.

Amyloid β deposition in areas of the brain linked to memory and cognition is always observed in AD patients. Early onset Alzheimer's was correlated to mutations in the gene encoding the precursor protein of $A\beta$, called amyloid precursor protein (APP) (SCHEUNER *et al.* 1996). Moreover, most people with trisomy 21 develop AD when ageing. Interestingly, the APP gene is found on chromosome 21 (KANG *et al.* 1987). Certain individuals with a specific mutation in their APP genes are less likely to develop AD, as this missense mutation reduces the production of APP (JONSSON *et al.* 2012). Neurofibrillary tangles cause by the hyperphosphorylation of the tau protein is another common finding in AD brain tissues. $A\beta$ oligomers cause the hyperphosphorylation of tau

protein, and also contribute to the presence of dystrophic neurites (JIN *et al.* 2011). Using a mouse model, amyloid β oligomers have also been found to affect synapse density, memory impairment, and inhibition of long-term potentiation of synaptic patterns (SHANKAR *et al.* 2008). *APOE*, gene which encodes for Apolipoprotein E, is the major risk gene for AD. The allele that increases the risk of developing AD (APOE-E4), decreases A β clearance rates in the brain (CASTELLANO *et al.* 2011).

3.2. Major genetic risk factor: *APOE* gene

Apolipoprotein E is one of the major components of high-density cholesterol (HDL) and is also found in chylomicrons, involved in the transport of cholesterol during triglyceride metabolism (BRESLOW *et al.* 1982; DAVIGNON *et al.* 1988; MAHLEY 1988). This protein is also believed to be involved in neuroplasticity and inflammation (KIM *et al.* 2009). *APOE*, the gene that encodes Apolipoprotein E, is found on chromosome 19 (OLAISEN *et al.* 1982), and has been associated with risk of developing AD (SAUNDERS *et al.* 1993).

From the three common *APOE* alleles (E2, E3 and E4) found within the human population, APOE-E4 (APOE4) is believed to be responsible for increasing the risk of AD (CORDER *et al.* 1993; STRITTMATTER *et al.* 1993). Corder and colleagues (1993) showed that individuals homozygous for the APOE4 allele have an eight-fold increased risk of developing late-onset AD. APOE4 gene dosage is inversely correlated to the age of onset of the disease (CORDER *et al.* 1993). Recently, with the aid of genome- wide association studies (GWAS), many other AD risk genes have been identified, although *APOE* still has the strongest effect on AD risk (HAROLD *et al.* 2009; KARCH AND GOATE 2015).

Correlation between the APOE4 allele and increased risk of developing AD is based on the impact that apolipoprotein E might have on A β homeostasis. An increased deposition of A β in the brains of APOE4 carriers, when compared to non-carriers, has been reported (REIMAN *et al.* 2009). In an *in vivo* study with mice, Castellano *et al.* (2011) showed that APOE4 affected A β clearance, generating a possible imbalance in A β homeostasis. Amyloid β deposition depended on the APOE isoform present (E4> E3> E2) (CASTELLANO *et al.* 2011). More recently, it has been proposed that *APOE* could regulate metabolism of A β indirectly, by interacting with transporters and receptors located on cell surfaces, for example the low-density lipoprotein (LDL) receptor protein 1 (LPR1) (VERGHESE *et al.*

2013). As an alternative mechanism for AD pathogenesis, it has been proposed that APOE4 could have a detrimental effect in tau protein processing (ANDREWS-ZWILLING *et al.* 2010).

3.3. *Antibiotics treatment*

We have known about AD for more than a hundred years; however, disease- modifying drugs, which completely block the progression of AD, are still being tested (KUMAR AND SINGH 2015). Acetyl choline esterase inhibitors (AChEIs) and N-methyl –D-aspartate (NMDA) receptor antagonists are currently prescribed to AD patients (ANAND *et al.* 2014). These drugs are aimed at alleviating certain symptoms of the disease but do not interfere with the pathology of the disease directly, and are not effective in many patients (FARLOW *et al.* 2008).

Research on A β vaccines, β -secretase and γ -secretase inhibitors, caspase inhibitors, metal (Cu-Zn) chelators, anti-inflammatory drugs, and nitric oxide synthase modulators, show certain potential (ANAND *et al.* 2014; KUMAR AND SINGH 2015). Nevertheless, clinical studies testing these treatments are time and resource intense, thus prolonging the discovery and approval of an urgently required optimal treatment for AD. Because of this, many researchers have changed their approach and have started to look at drugs used for other conditions, which might have the potential to treat AD patients efficiently (CORBETT *et al.* 2012).

Following this drug repurposing strategy, researchers have found that some antibiotics have the potential to be used for AD treatment. Doxycycline and Rifampicin have shown to have certain potential as therapeutic alternatives for patients with mild to moderate AD (LOEB *et al.* 2004). However, clinical data is still very limited and there is no epidemiological evidence as of yet (CORBETT *et al.* 2012). Minocycline could potentially be used to treat AD also. Minocycline has been shown to decrease the total number of microglia and astrocytes in rat brains induced with A β , as well as a reduction of neurological loss (RYU *et al.* 2004). Fan *et al.* (2007) were not able to show a significant change in the deposition of A β in mice brains after four weeks of minocycline treatment, however, they observed an improvement in cognitive behavioural performance and a decrease in microglial activation (FAN *et al.* 2007). Moreover, using *in vivo* and *in vitro*

models, researchers have found that minocycline treatment can prevent the aggregation of hyperphosphorylated tau proteins (NOBLE *et al.* 2009a).

Minocycline is a lyophilic bacteriostatic antibiotic derived from tetracycline (NOBLE *et al.* 2009b). This drug is readily absorbed by the cerebrospinal fluid and the central nervous system (CNS) (KLEIN AND CUNHA 1995). It is considered as a neuroprotective agent because of its involvement in important anti-inflammatory and anti-apoptotic pathways, especially in the CNS (STIRLING *et al.* 2005). Nevertheless, its specific mechanism of action as a therapeutic for AD is not yet clear and clinical and epidemiological evidence is still required (CORBETT *et al.* 2012).

3.4. Pathogen hypothesis

The pathogen hypothesis also attempts to explain the pathogenesis of AD. By RT-PCR and immunohistochemical examinations, Bailin *et al.* (1998) concluded that *Chlamydia pneumoniae* (Cpn) was present, transcriptionally active and viable, in specific areas of the brain associated with AD (BALIN *et al.* 1998). These findings were later confirmed by Gérard *et al.* (2004). In that same year, a study showing the development of AD-like symptoms in mice infected with Cpn was conducted (LITTLE *et al.* 2004). Guided by Koch's Postulates, researchers infected BALB/c mice with Cpn, previously isolated from AD brains, and observed increased amyloid deposition in mice brains as the bacterial infection progressed, up to 3 months post-infection (LITTLE *et al.* 2004).

Other microorganisms, such as *Herpes simplex virus* (ITZHAKI *et al.* 1997; DOBSON AND ITZHAKI 1999; LIN *et al.* 2002; ITZHAKI 2018), different *Spirochetes* (MACDONALD 2006; MIKLOSSY 2011a), and *Helicobacter pylori* (WANG *et al.* 2014a) have also been linked to AD, providing more support for the pathogen hypothesis. After Balin and colleagues (1998) isolated Cpn from AD brains, others attempted to confirm the connection between Cpn and AD. While some managed to provide evidence for the involvement of this bacterial species in AD (LITTLE *et al.* 2004; GÉRARD *et al.* 2006; DRESES-WERRINGLOER *et al.* 2009; HAMMOND *et al.* 2010; LITTLE *et al.* 2014), others were not as successful (NOCHLIN *et al.* 1999; GIEFFERS *et al.* 2000; RING AND LYONS 2000; TAYLOR *et al.* 2002; BOELEN *et al.* 2007). Variability in virulence and tissue tropism of different strains of Cpn might be the reason for their failed attempt at showing a relationship between Cpn and AD (LITTLE

et al. 2014). Cpn isolated from AD brains and respiratory strains, such as AR39, present important differences in certain gene families (e.g. *IncA*, *pmp*, and *tyrP*) involved in virulence (ROULIS *et al.* 2015). Roulis and colleagues (2015) also noted that these differences could be related to different immune responses from the host.

As bacteria and other microorganisms may be linked to AD pathogenesis and pathophysiology, attention is drawn to the involvement of the human microbiome in this disease. A possible connection between the gut microbiome and AD has been proposed (BHATTACHARJEE AND LUKIW 2013; HILL *et al.* 2014a; HILL *et al.* 2014b; HILL AND LUKIW 2015; SHOEMARK AND ALLEN 2015; ZHAO AND LUKIW 2015; PISTOLLATO *et al.* 2016). The gut-microbiome could be linked to AD pathogenesis through the brain-gut axis, for example by secreting molecules that could modulate the central nervous system (CNS) chemistry (BHATTACHARJEE AND LUKIW 2013; HILL *et al.* 2014a). Moreover, the dysbiosis of microbial communities could contribute to the dyshomeostasis of amyloid proteins (HILL AND LUKIW 2015; ZHAO AND LUKIW 2015; PISTOLLATO *et al.* 2016). Shoemark and Allen (2015) explored the potential link between the oral microbiome and AD, emphasising the importance of the immune system, aging, and inflammation, as important factors to understand the role of the human microbiome in AD pathogenesis and progression (SHOEMARK AND ALLEN 2015). Even though sparse attention has been given to the possible link of the gut and oral microbiomes and AD, no research has investigated whether there is a relationship between AD and the blood microbiome.

4. Aim and Objectives

4.1. *Aim:*

The aim of this study is to investigate the blood microbiome in Alzheimer's patients and healthy individuals, with an emphasis on AD risk factors and the blood environment.

4.2. *Objectives:*

1. Investigate the bacterial composition and diversity in the blood of Alzheimer's patients and healthy individuals.
2. Study differences in the blood microbiome between AD patients, their cohabitants, as well as young and elderly healthy control groups.
3. Determine whether AD risk factors (sex, ABO blood group and *APOE* genotype) and haematological parameters (serum Fe, white- and red blood cells) have an effect on the blood microbiome of AD and healthy individuals.

Chapter 2

Exploring the blood
microbiome in Alzheimer's
disease and healthy
individuals

Chapter 2

Title page

Exploring the blood microbiome in Alzheimer's disease and healthy individuals

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Original research article

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1. Abstract

Human blood has traditionally been considered a sterile environment. However, evidence in support of a bacterial component in the blood of healthy individuals is steadily accruing. Dysbiosis of this blood-microbiome has been linked to various diseases, *i.e.*, diabetes, cirrhosis and liver- and cardiovascular-disease. Alzheimer's disease (AD) is an important neurodegenerative condition which, causes dementia in the elderly. According to the pathogen hypothesis, microorganisms may play an important role in the pathogenesis of AD. While the intestinal microbiome could be linked to the onset of AD, the relationship between AD and the blood microbiome remains unexplored. In this study, we performed comparative analyses of the blood microbiome in AD and healthy human individuals, placing an emphasis on AD risk factors and the blood microbial environment, via amplicon and metagenomic sequencing of the 16S rRNA gene. Results indicate that bacterial DNA is present in the blood of both healthy individuals and in AD patients. However, the concentration of bacterial DNA does not differ in Alzheimer's patients, when compared to healthy participants. Whereas AD patients presented a higher alpha-diversity than healthy participants, beta-diversity did not differ between the groups. Certain haematological factors that represent the blood environment appears to influence bacterial taxonomy and diversity in the blood. Conversely, AD associated risk factors, such as the *APOE* genotype, does not influence bacterial community structure or taxonomy in the blood of AD patients. Future research should aim to explore the link between the human blood microbiome and the immune system, as well as the relationship between bacterial communities in the blood and those found in the intestinal tract, skin and the oral microbiome.

2. Introduction

Human microbiomics is a developing field of research which deals with microorganisms that live in association with the human body. This discipline focuses on the impact that microorganisms have on human physiology and health (RAJENDHRAN AND GUNASEKARAN 2010). Studies on human gut microbiomes have provided us with information regarding different possible roles of the human microbiome in health and disease. The human gut microbiota have been shown to play important roles in their hosts, such as the participation in normal metabolism (nutrition, digestion, and growth), the immune system, and brain development (FORSYTHE *et al.* 2012; DOUGLAS-ESCOBAR *et al.* 2013). Even though the human gut microbiome has been described in more detail, microbial communities, and especially bacteria, have also been identified in the oral cavity, eyes, lungs, skin and urogenital tract (Lloyd-Price *et al.* 2016).

In the study of the human microbiome and disease, the concept of dysbiosis is especially important. Dysbiosis refers to a change in the composition of symbiotic or commensal microbial communities in diseased patients, when compared to those in healthy individuals (Petersen and Round 2014). Even though it is not known whether dysbiosis is a cause or simply a reflection of a diseased state in a patient (Bäckhed *et al.* 2012), many studies have linked changes in human microbial communities with disease. These include conditions such as diabetes (Qin *et al.* 2012), asthma (Teo *et al.* 2015), inflammatory bowel disease (Morgan *et al.* 2012), autism (Parracho *et al.* 2005), and Alzheimer's disease (AD) (Pistollato *et al.* 2016). Although many studies have addressed the link between the gut-microbiome and disease, very little is known about dysbiosis of microbial communities that live in association with the human blood and its potential connection to disease.

The blood microbiome is a novel concept, still surrounded by some controversy (McLAUGHLIN *et al.* 2002; MITCHELL *et al.* 2016). However, research supporting the existence of bacteria in the blood of healthy individuals keeps accumulating, especially in the past three years (GOSIEWSKI *et al.* 2016; PAÏSSÉ *et al.* 2016; KOWARSKY *et al.* 2017; TRAYKOVA *et al.* 2017; LI *et al.* 2018). Although these studies are mostly

supported by the finding of bacterial DNA in the blood, microscopy (McLAUGHLIN *et al.* 2002; PANAIOTOV *et al.* 2018), and RNA-seq data (LOOHUIS *et al.* 2018) also point towards the existence of a healthy human blood microbiome. Notwithstanding this evidence, the concept of microorganisms inhabiting human blood is difficult to accept, as leukocytes and other immune system response effectors are routinely transported via the blood (JERNE 1973; SPRINGER 1990). Different subtypes of white blood cells involved in the innate and acquired immune responses travel freely through the blood (JERNE 1973; PORTH 2004). This environment also house red blood cells, which ensures oxygen transportation throughout the body (ALBERTS *et al.* 2002). Changes in blood bacterial composition could be linked to a disruption of the balance between bacteria in the blood and the immune system. Dysbiosis of blood microbiome in diabetes (AMAR *et al.* 2011; SATO *et al.* 2014), cardio vascular disease (AMAR *et al.* 2013; DINAKARAN *et al.* 2014), cirrhosis (TRAYKOVA *et al.* 2017), pancreatitis (LI *et al.* 2018), and liver disease (LELOUVIER *et al.* 2016) has been reported. Nonetheless, the relationship between the blood microbiome and AD remains to be explored.

The most common form of this neurodegenerative disorder is late-onset AD, which usually affects people over the age of 65 (SELKOE 2001). Even though AD was discovered more than a hundred years ago, our knowledge about its pathogenesis and progression is rather limited (SELKOE AND HARDY 2016). The most relevant clinical findings in post-mortem brain consist of senile plaques and neurofibrillary tangles. Senile plaques are aggregates of the protein amyloid β , which causes inflammation and neuronal loss when not cleared correctly (GLENNER AND WONG 1984; SCHEUNER *et al.* 1996). On the other hand, neurofibrillary tangles are aggregates of phosphorylated tau protein, detrimental to neuronal health (SELKOE 2001; JIN *et al.* 2011). APOE is the major risk gene linked to late-onset AD (SAUNDERS *et al.* 1993). Three different alleles, consisting of a combination of two SNPs, are known for this gene (APOE - E2, E3, and E4). Allele E4 increases the risk of developing AD (CORDER *et al.* 1993; STRITTMATTER *et al.* 1993), while E2 seems to have a protective effect against it (WU AND ZHAO 2016). AD is a very complex disorder and other risk factors, such as sex and ABO blood group, have also been linked to this disease. Women are more prone to develop AD than men (LAMBERT *et al.* 2004; VINA AND LLORET 2010),

while the O blood type could translate into a lower risk of acquiring neurological conditions (DE MARCO AND VENNERI 2015).

According to the pathogen hypothesis, microorganisms may also be involved in AD pathogenesis and progression. For example, *Chlamydia pneumoniae* has been isolated from AD post-mortem brains (BALIN *et al.* 1998). Moreover, mice infected with this bacterial species developed AD-like symptoms (LITTLE *et al.* 2014). *Helicobacter pylori* (WANG *et al.* 2014a), different *Spirochetes* (MACDONALD 2006; MIKLOSSY 2011a), and *Herpes simplex* virus (ITZHAKI *et al.* 1997; DOBSON AND ITZHAKI 1999; LIN *et al.* 2002; ITZHAKI 2018), have been found in Alzheimer's patients. Not only pathogenic bacteria may be involved in AD pathogenesis and development. Commensals from the gut microbiome might play a role in AD, through the brain-gut axis, by for example secreting molecules that modulate the central nervous system chemistry (BHATTACHARJEE AND LUKIW 2013; HILL *et al.* 2014a; HILL *et al.* 2014b; HILL AND LUKIW 2015; SHOEMARK AND ALLEN 2015; ZHAO AND LUKIW 2015; PISTOLLATO *et al.* 2016). Moreover, dysregulation of amyloid protein clearance, a hallmark of AD, could be linked to dysbiosis of microbial communities in the gut (HILL AND LUKIW 2015; ZHAO AND LUKIW 2015; PISTOLLATO *et al.* 2016). Even though the gut microbiome has been linked to AD, the possible relationship between the human blood microbiome and AD remains to be elucidated.

The aim of this research was to study the blood microbiome in Alzheimer's and healthy individuals, with an emphasis on AD risk factors and the blood environment. This was achieved by investigating the bacterial composition and diversity in AD patients and healthy individuals. The possible differences between the blood microbiome in AD patients, their cohabitants, and healthy control groups was explored. Finally, we investigated whether AD risk factors (sex, ABO blood group and *APOE* genotype) and haematological parameters (serum Fe, white- and red blood cell) had an effect on bacterial taxonomy and diversity in the blood healthy individuals.

3. Materials and methods

3.1 *Participants and ethical clearance*

Prior to commencement of the study ethics approval was obtained from the Human Research Ethics Committee, Faculty of Health Sciences, University of Pretoria. Approval was granted for 4 years under protocol number 27/2017. Approval from the Faculty of Natural and Agricultural Sciences Ethics Committee (University of Pretoria) was also obtained (EC170530).

Study participants were divided into four different groups: 1) Alzheimer's disease (AD) patients, 2) AD patients' cohabitant care-givers, 3) old control group (65-80 years old), and 4) young control group (20-35 years old). Study groups were named after four letters of the alphabet (X, Y, W and V, following the order described earlier). Twenty participants were recruited in each group. Participants with AD and their cohabitant care-givers were recruited, in collaboration with Dr. Mariët van Niekerk, expert neurologist at NHC Medical Centre, Centurion, South Africa. During patient consultation Dr. van Niekerk determined whether the patient pair (patient and cohabitant caregiver) conformed to the inclusion criteria to participate in the study. Following a positive diagnosis with AD the patient pair were approached by Dr. van Niekerk to enquire about their willingness to participate. When patient pairs agreed, the principle investigator of the study consulted with the patient pair at their home to give detailed information about the study during which they could make an informed decision about their voluntary participation. If they agreed, they were asked whether more information or details about the study was required. Once the study leader was comfortable that the participant is familiar with the requirements, aims and objectives of the research the study participants were asked to give written consent for their participation. In the case of patients with Alzheimer's disease, a short test was conducted to confirm that they understood what the study entails (see Apendix). If the patient passed the test, he or she was allowed to sign their own consent form, always in the presence and with the approval of their cohabitant caregiver. If the study leader was not convinced that the patient completely understood the information but still wanted to participate in the research, the cohabitant, spousal caregiver were asked to sign on behalf of the patient. After consent was obtained, blood was collected from the participants by a

registered nurse. Healthy control participants were recruited through the extended personal and professional networks of the research team. Candidates were presented with detailed information about the study and asked whether they wanted to participate. The same informed consent process as for the AD patient pairs were followed before blood collection was done.

General exclusion criteria for all groups was the use of antibiotics less than three months prior to participating in the study, suffering from type I diabetes, or any prior diagnosis with an autoimmune disease. Regarding inclusion criteria; Group X: AD patients had to be between of 65 to 85 years old, of either sex, with a positive diagnosis of Alzheimer's dementia, by Mini State Examination (MMSE), electroencephalogram (EEG), and brain imaging if needed. Group Y: spousal care-givers needed to have lived with the patient for more than 10 years. Group W: Healthy elderly participants had to be between the ages of 65 to 80 years old, with an MMSE score representing normal cognition, and self-reported as being healthy. Group V: Healthy young participants was limited to self-reported healthy couples, between the ages of 20 and 35 years old, who have lived together for more than a year.

3.2 Blood collection and negative controls

Participants donated 15 ml of blood for the research experiments. Standard venepuncture was performed by an expert phlebotomist after thoroughly cleaning the skin was with 70% (v/v) alcohol wipes. Serum Iron and haematology tests (section 1.3), as well as bacterial DNA extraction (section 1.5.1), were performed on the same day of blood collection. Thereafter the blood was frozen at -80 C° until the extraction of genomic DNA for APOE genotyping (section 1.4.1.). Appropriate strict negative controls were included to control for contamination during the blood collection procedure.

Potential low-biomass microbial studies require the use of strict negative controls (NTCs). In this study 15 NTCs were included for all the steps leading to the study of bacterial DNA present in human blood (Fig. 1). Five NTCs served to control for possible contamination from the skin during the blood extraction procedure. For these control samples, the skin was cleaned as was done for normal venepuncture, the skin was then touched with the needle without puncturing it. The needle tip was rinsed in 5 ml of sterile water (Water for

Molecular Biology, by Merck) in an EDTA collection tube. To test for potential contamination from sterile needles, the same process was performed with the needles, but this time with no contact with the skin. Water from these eight tubes was taken through the normal process of bacterial DNA extraction (see section 1.5.1). This process was performed three times. To test for possible contamination during the DNA extraction protocol itself, 5 ml of the same of Water for Molecular Biology (Merck) was pipetted into a 50 ml conical Falcon centrifuge tube and DNA isolation was performed, in triplicate. The other six negative controls, three samples for each, consisted of assessing potential contamination from distilled water (ddH₂O) provided by kit, and sterile water used in all the other controls (Water for Molecular Biology, by Merck). For each type of water, 80µl was pipetted into three separate 1.5 microcentrifuge tubes (Eppendorf, Germany) (six in total), for later analysis by qPCR for bacterial DNA quantitation (see section 1.5.2), and 16S rRNA next generation amplicon sequencing (see section 1.5.3).

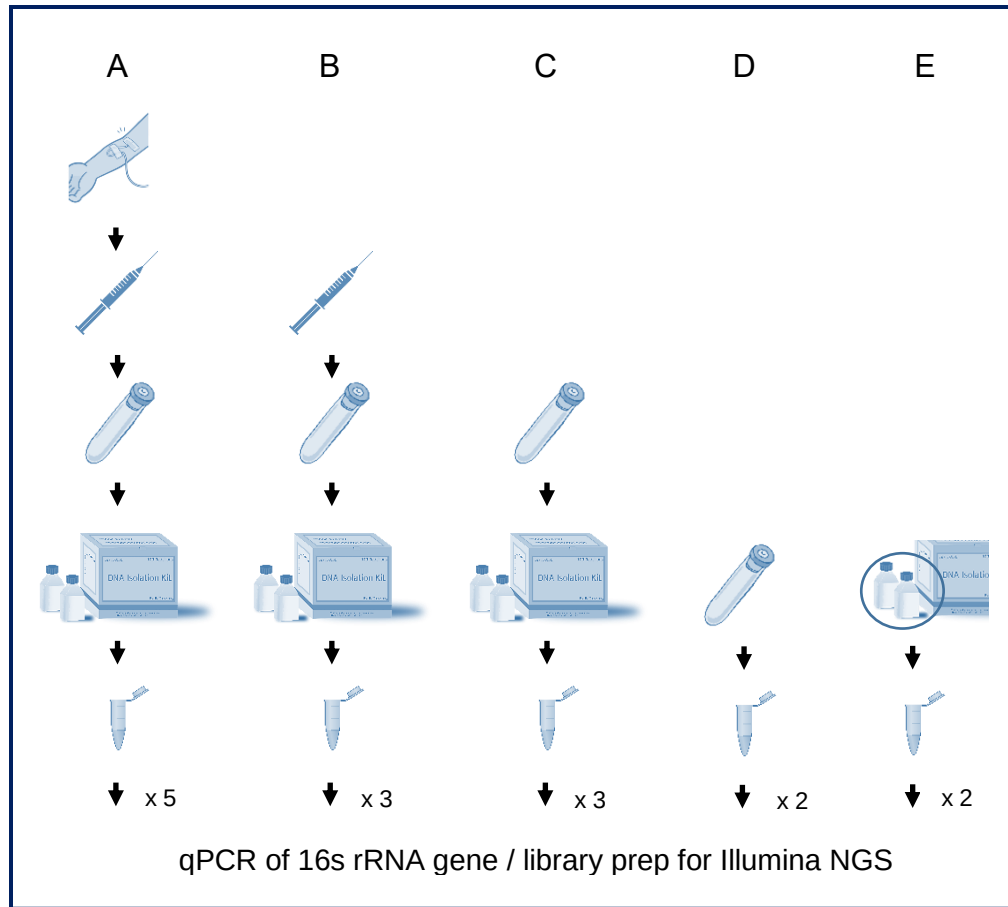


Figure 1. Graphic representation of 15 negative controls (NCTs) included in the study to controls for possible confounding effects when studying blood bacteria. For each set of controls, a number after the times symbol (x), next to the last arrow, indicates how many times the whole process was repeated. **A**, negative control for skin. Sterile needle was used to touch the skin of the forearm after sterilising it, then the same needle was used to absorb molecular biology (MB) sterile water, and DNA isolation was performed. **B**, NTC for needle. MB water was absorbed with sterile needle and DNA isolation protocol was carried on. **C**, control for DNA isolations procedure (reagents, consumables, environment, etc.). MB water was used directly to perform DNA isolation procedure. **D**, NTC to control for MB water. Molecular biology sterile water used in A, B, and C, was sent for sequencing directly. **E**, control for kit double distilled water (dH₂O). Eighty microliters of dH₂O from the kit was pipetted into a 1.5 microcentrifuge tubes (Eppendorf, Germany) and sent for qPCR of 16S rRNA gene and library preparation for next generation (NG) amplicon sequencing in Illumina MiSeq.

3.3 *Serum Iron and haematology tests*

Serum iron concentration ($\mu\text{mol/l}$) was measured by AMPATH laboratories, South Africa. Haematology analyses were performed by the National Health Laboratory Service (NHLS), Pretoria, South Africa. Full blood counts, differential blood counts, ABO blood type, and Resus factor were determined for all participants. The following parameters were measured for full blood count: white cell count ($\times 10^9/\text{l}$), red cell count ($\times 10^{12}/\text{l}$), haemoglobin concentration (g/dl), haematocrit (l/l), mean corpuscular volume (MCV) (fl), mean corpuscular haemoglobin (MCH) (pg), mean corpuscular haemoglobin concentration (MCHC) (g/dl), red cell distribution width (%) and platelet count ($\times 10^9/\text{l}$). Differential counts consisted of the measurement of neutrophils, lymphocytes, monocytes, eosinophils, and basophils; both as percentages and as a total count ($\times 10^9/\text{l}$).

3.4 *APOE genotyping using qPCR*

3.4.1 *Genomic DNA extraction from whole blood*

Genomic DNA extraction from whole blood was performed using the QIAamp DNA Mini Kit (QIAGEN, Germany). Two hundred microliters of blood was used for the DNA isolation, according to manufacturer's instructions (QIAGEN 2016). Briefly, the protocol is based on spin and vacuum steps which rapidly yield pure DNA. After lysis with proteinase K, purification on mini spin columns was performed with provided binding, washing and elution buffers. Sterile water (Sabax) was used as starting material for negative controls. After the isolation of total genomic DNA, agarose gel electrophoresis 1% (w/v) was performed and visualised with the GelDoc™ XR+ Imaging System. The DNA concentration was confirmed by High Sensitivity fluorometer analysis using the Qubit 2.0 Fluorometer (ThermoFisher Scientific, USA).

3.4.2 *Genotyping of APOE gene by qPCR*

Genotyping of the APOE gene was done using quantitative polymerase chain reaction (qPCR) and TaqMan Probe (ThermoFisher Scientific, USA) technology. Probes targeting the two most common single-nucleotide polymorphisms (SNPs) that confer the different APOE genotypes were used (Table 1). The TaqPath ProAmp Master Mix was used to set up the PCR reactions in 96 well plates, which were prepared according to the

manufacturer's instructions (APPLIEDBIOSYSTEMS 2016). For negative controls, no DNA was added to the wells. Reactions were prepared in triplicates, and in two separate sets, one per TaqMan assay, after the DNA concentration of each sample had been standardised to 10 ng/ml (Table 2).

Table 1: TaqMan probes and SNP assay information

Assay ID	RefSNPs number	NCBI SNP Reference	SNP Type	A. Acid Change	Context sequence
C__904973_10	rs7412	APOC1;TOMM40; APOE	Missense mutation	R176C	CCGCGATGCCGATGACCTGCA GAAG[C/T]GCCTGGCAGTGTAC CAGGCCGGGGC
C__3084793_20	rs429358	APOC1;TOMM40;APOE	Missense mutation	R130C	GCTGGGCGCGGACATGGAGGA CGTG[C/T]GCGGCCGCCTGGTG CAGTACCGCGG

Table 2: PCR reactions set up for genotyping of APOE gene rs7412

Component	Volume per reaction
TaqPath ProAmp Master Mix	5.0 µl
TaqMan SNP assay	0.25 µl [20x]
Genomic DNA	4.5 µl
Nuclease Free water	To to 10 µl total
Total volume	10 µl

Quantitative PCR was then performed in the QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific), at the University of Pretoria, according to standard cycling for genotyping, as per the manufacturer's instructions (Table 3). Allelic discrimination was then performed with the use of TaqMan Genotyper Software for APOE status assignment.

Table 3: Real-time PCR genotyping under standard conditions

Step	Cycles	Time	Temp. (C°)
Pre-read	Hold	30 seconds	60
Initial denature		5 minutes	95
Denature	40	15 seconds	95
Anneal		60 seconds	60
Post-Read	Hold	30 seconds	60

3.5 *Bacterial DNA studies*

3.5.1 *Bacterial DNA extraction from whole blood*

Bacterial DNA isolation from whole blood was performed using the MoLYsis™ Complete5 kit (Molyzym, Germany). The technology allows removal of human DNA prior to isolation of microbial DNA in the same sample. The protocol was performed according to the manufacturer's instructions (MOLYZYM 2014). In brief, 5 ml of fresh blood was used as starting material. A chemotropic buffer was added to the samples to lyse all human cells, while the bacterial DNA remained intact. The enzyme MolDNAse B was then used to degrade the DNA released from human cells. Thereafter Gram-negative and Gram-positive cell walls were degraded by treatment with BugLysis reagent, followed by digestion by Proteinase K. Finally, the bacterial DNA was extracted and isolated by a bind-wash-elute procedure. The concentration of the bacterial DNA isolated was then studied by High Sensitivity fluorometer analysis using the Qubit 2.0 Fluorometer (ThermoFisher Scientific). Strict negative controls were also included for the extraction and isolation of bacterial DNA (see section 3.2. on blood collection and negative controls).

3.5.2 *Bacterial DNA quantitation by qPCR*

All samples, including the 15 NTCs were analysed by qPCR, to determine the approximate number of 16S copies per µl of bacterial DNA extracted from whole blood. This analysis was performed in collaboration with Molecular Research (MR) DNA laboratory (Shallowater, Texas, USA) The quality and quantity of bacterial DNA was determined using a NanoDrop 200 (ThermoFisher Scientific). A total volume of 1 µl of DNA per sample was used to set up the PCR reactions, using TaqMan Universal Master Mix (Applied Biosystems), according to manufacturer's instructions. Three replicates from each sample were included. MR DNA Bacteria 2F and Bacteria 2R primers, specific for 16S rRNA region, were used for the reactions (Dowd 2017). Quantitative PCR was performed in StepOnePlus Real-Time PCR platform (Applied Biosystems), according to the following cycling parameters: an initial incubation stage of 50 °C for 2 minutes, followed by 95 °C for 10 minutes, for polymerase activation; then 40 cycles of 95 °C for 15 seconds, 55 °C for 15 seconds, and 60°C for 1 minute were performed. As a standard, DNA from *E. coli* was used.

3.5.3 16S rRNA next generation sequencing

Next-generation amplicon sequencing of the 16S rRNA gene was done in collaboration with Molecular Research (MR DNA) Laboratory (Shallowater, Texas, USA). The V3-V4 hypervariable region of the 16S rRNA gene was sequenced by pair-end sequencing, with the primers 341F and 785R, using the Illumina (MiSeq) technology. First, PCR was completed using the HotStarTaq Plus Master Mix Kit (Qiagen), according to the manufacturer's instructions. The cycling conditions consisted of an initial denaturation step at 94 °C for 3 minutes; 30 cycles at 94 °C for 30 seconds, 53 °C for 40 seconds, and 72 °C for 1 minute; and a final elongation step for 5 minutes at 72 °C. Thereafter, the amplified products were analysed by agarose gel (2% (v/v)) electrophoresis. The 15 negative controls (see section 1.2.) were also included in this analysis, nine of which were taken to the next step. PCR products belonging to multiple samples were pooled together in equal proportions when they presented similar molecular weight and DNA concentration. At that point Ampure XP beads were used in order to purify the pooled samples, followed by preparation of the Illumina DNA library. Finally, the MiSeq platform was used to perform end-paired sequencing, following the manufacturer's guidelines (Dowd 2017).

3.6 Data processing and statistical analysis

The raw sequencing reads generated by the 16S rRNA sequencing protocol were analysed using Quantitative Insights Into Microbial Ecology (QIIME) I and II (CAPORASO *et al.* 2010). *Fasta* and *quality* files were combined into *fastq* files. Because the sequencing was done in separate runs, two nucleotides were added to the barcodes, before pooling all reads together. After the files were combined, barcodes were extracted and artefacts were created for processing in QIIME II. The sequencing reads were demultiplexed according to the specific barcodes belonging to each sample, including the nine negative controls (NTCs). At this point, sequence quality control and construction of feature and representative sequences tables was performed, using the DADA2 plugin (CALLAHAN *et al.* 2016). In this step the barcodes were removed from the sequences and the reads were trimmed to be of a maximum length of 295 nucleotides. Representative sequences were aligned using MAFFT algorithm (KATO AND STANDLEY 2013), and highly

variable regions were masked to reduce noise for phylogenetic analysis. A phylogenetic tree was generated and rooted at midpoint. Finally, taxonomic classification based on the SILVA database (QUAST *et al.* 2012) 97 % classifier was achieved, using the feature table. In Linux, all reads OTUs present in the nine negative-control samples were removed from the taxonomy and OTU table files. The taxonomy, feature table (OTU table), and tree files were exported for further analysis in R software.

Taxonomy, OTU table, tree- and metadata files were imported into R for microbial analysis. A metadata file containing 16S copy numbers, APOE status, blood type, serum iron levels, and blood counts, was also imported into R. The phyloseq package (McMURDIE AND HOLMES 2013) was used for microbial community analysis . Other R-based packages such as ggplot2, vegan, and microbiome-seq were also used. Before performing statistical analysis, all samples were rarefied at 645 reads. The number of samples per group was standardised for comparison between different groups. We first, analysed if the different blood parameters measured were significantly different between the four study groups by analysis of variance (ANOVA). Analysis of bacterial abundance at different taxonomic levels was then performed, and significant differences between groups was studied using Kruskal-Wallis one-way analysis of variance and DESeq2 algorithm. Potential correlations of taxa abundance to independent continuous variables was studied using Pearson's method. Alpha and beta diversity were studied using phyloseq. ANOVA was used to work out differences in alpha diversity between the four study groups. To study beta-diversity we constructed non-metric multidimensional scaling (NMDS) and principal coordinate analysis (PCoA) plots, based on different distances metrics (weighted and unweighted UniFrac, Bray-Curtis, and Jaccard index). Permutational multivariate analysis of variance (PERMANOVA) was calculated to explore differences observed in beta diversity between groups. Pearson's correlation was also performed to study possible relationships between diversity indices and blood parameters measured.

4. Results

4.1 Iron levels and blood cell counts

Serum Iron levels and blood counts data were obtained from most participants (Groups X, Y, V and W) (Table A1). Serum Fe levels could not be gathered from two patients of AD group (X), and three patients of the AD co-habitants healthy group (Y), due to insufficient blood volume collected. All parameters measured for full blood count, differential blood count, and serum Fe levels were analysed to determine if there were significant differences between groups.

Haemoglobin concentration, red blood cell distribution width, neutrophil and lymphocytes differential counts showed a significant difference between all groups (Table 4; ANOVA p-value < 0.05). These parameters were further examined by Tukey's test.

Table 4: ANOVA and Tukey's test p-values of blood count parameters and serum Fe levels.

Parameter	ANOVA p-value	Paired comparison significant p-values (Tukey's test)
Serum Fe	0.6760	
White cell counts	0.4310	
Red cell counts	0.0772	
Haemoglobin	0.0375 *	Y – V: 0.0420
Haematocrit	0.0956	
Mean corpuscular volume	0.1520	
Mean corpuscular haemoglobin	0.8030	
Red cell distribution width	0.0021 *	X – V 0.0046 Y – V 0.0096
Platelet count	0.2360	
Neutrophils	0.0148 *	X – V 0.0010
Lymphocytes	0.0020 *	X – V 0.0015 Y – V 0.0208
Monocytes	0.1470	
Eosinophils	0.7500	
Basophils	0.1190	

Significance key: *0.05. X, AD patients; Y, AD co-habitants; V, young control group.

The young control group (V) showed significantly higher haemoglobin concentration and lymphocyte count, when compared to the AD-cohabitant group (Y) (Figure 2). Lymphocyte count was also significantly higher in young individuals when compared with that of AD patients. Young healthy participants showed significantly lower red blood cell distribution width and neutrophil counts than AD patients. Young individuals also presented with a lower red cell distribution width percentage when compared with AD-

cohabitants (group Y). It is interesting to note that the only significant differences in neutrophil differential counts were observed between young healthy individuals (group V) and Alzheimer's patients (group X), and not between young healthy individuals (group V) and the other (healthy) elderly control groups (W and Y).

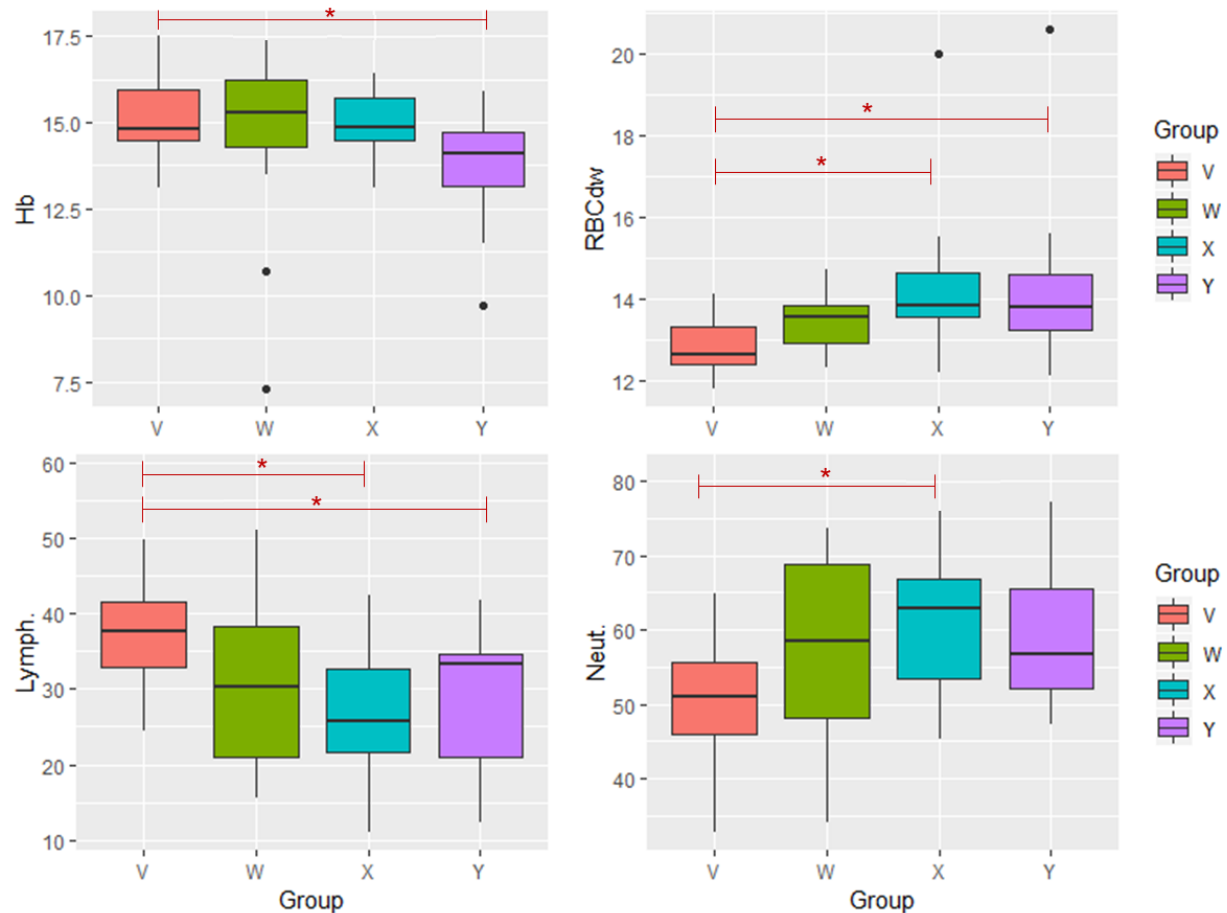


Figure 2. Box-plots of blood parameters showed a significant difference between groups (ANOVA, p-value < 0.05). Group V, young healthy controls; W, old healthy controls; X, AD patients; Y, AD healthy cohabitants. Hb, haemoglobin concentration (g/dl); RBCdw, red cell distribution width (%); Lymph., lymphocyte count ($\times 10^9/l$), Neut., neutrophil count ($\times 10^9/l$).

4.2 APOE genotyping using qPCR

Quantitative PCR using TaqMan probes (ThermoFisher Scientific) was performed to obtain the APOE genotype of all participants. DNA concentrations ranged from 15 to 55 ng/ μ l, and negative controls on agarose gel did not present bands. After qPCR procedure, allelic determination was successful for all participants using the TaqMan Genotyper

Software (Table A2). The prevalence of different genotypes and alleles are summarised in Tables 5 and 6.

Table 5: Summary of prevalence of APOE genotypes in the four study groups

Groups	Young control (V)		Old control (W)		AD patients (X)		AD-cohabitants (Y)		Total	
Genotypes	count	%	count	%	count	%	count	%	count	%
E2/E2	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
E2/E3	3	15.00%	4	20.00%	2	10.00%	3	15.79%	12	15.19%
E2/E4	0	0.00%	1	5.00%	0	0.00%	1	5.26%	2	2.53%
E3/E3	11	55.00%	10	50.00%	7	35.00%	8	42.11%	36	45.57%
E3/E4	6	30.00%	3	15.00%	6	30.00%	6	31.58%	21	26.58%
E4/E4	0	0.00%	2	10.00%	5	25.00%	1	5.26%	8	10.13%
Total	20	100%	20	100%	20	100%	19	100%	79	100%

Table 6: Summary of prevalence of different APOE genotypes in four study groups

Groups	Young control (V)		Old control (W)		AD patients (X)		AD-cohabitants (Y)		Total	
Alleles	count	%	count	%	count	%	count	%	count	%
E2	3	7.50%	5	12.50%	2	5.00%	4	10.53%	14	8.86%
E3	31	77.50%	27	67.50%	22	55.00%	25	65.79%	105	66.46%
E4	6	15.00%	8	20.00%	16	40.00%	9	23.68%	39	24.68%
Total	40	100 %	40	100 %	40	100 %	38	100 %	158	100 %

As expected, E3/E4 and E4/E4 genotypes were more prevalent in the AD group. When all healthy participants were compared to all AD patients, it was found that there was a relationship of dependency between E4/E4 genotype and suffering from AD (Fisher exact test, p-value 0.019). A relationship between those variables was also observed at the allelic level (chi-squared, p-value 0.035).

4.3 Bacterial DNA in the blood of AD and healthy individuals

4.3.1 Extraction of bacterial DNA from blood

In order to study bacterial DNA from human blood, a DNA extraction procedure was performed on freshly collected blood. Microbial DNA from blood of all participants was extracted and isolated using the MoLYsis™ Complete5 (Molyzym, Germany) kit. This process ensured the removal of human DNA before isolating microbial genetic material

(MOLYZYM 2014; VUTUKURU *et al.* 2016; MARIN *et al.* 2017). The quality and concentration of the extracted DNA was then analysed by High Sensitivity fluorometer Qubit analysis (ThermoFisher Scientific). Concentration values ranged from 0.10 to 5.25 ng/μl. As expected when working with low biomass microbiomes (PAÏSSÉ *et al.* 2016; TRAYKOVA *et al.* 2017), these values were very low and concentrations varied over a wide range, due to inter-individual differences in blood bacteria content. The low DNA concentrations obtained gave a possible indication that the host's DNA was successfully digested before microbial DNA extraction and isolation. All 15 negative controls gave a negative results (unable to measure a value on the Qubit due to low concentration). The samples were analysed by qPCR (next section); and standard PCR, followed by agarose gel electrophoresis, before sequencing.

4.3.2 Quantitation of bacterial DNA

The estimated 16S rRNA gene copy numbers present in human blood of AD patients and healthy individuals was investigated using real-time qPCR. The aim was to quantify bacterial DNA in all study participants. Fifteen different negative controls (NTCs), designed to detect potential contamination from the skin, needle, DNA extraction reagents and consumables, *etc.* were also tested. The findings are summarized in Figure 3 and Table 7, below. The estimated 16S rRNA gene copy numbers per μl of blood for all participants and 15 negative controls can be found in the Appendix section (Table A3).

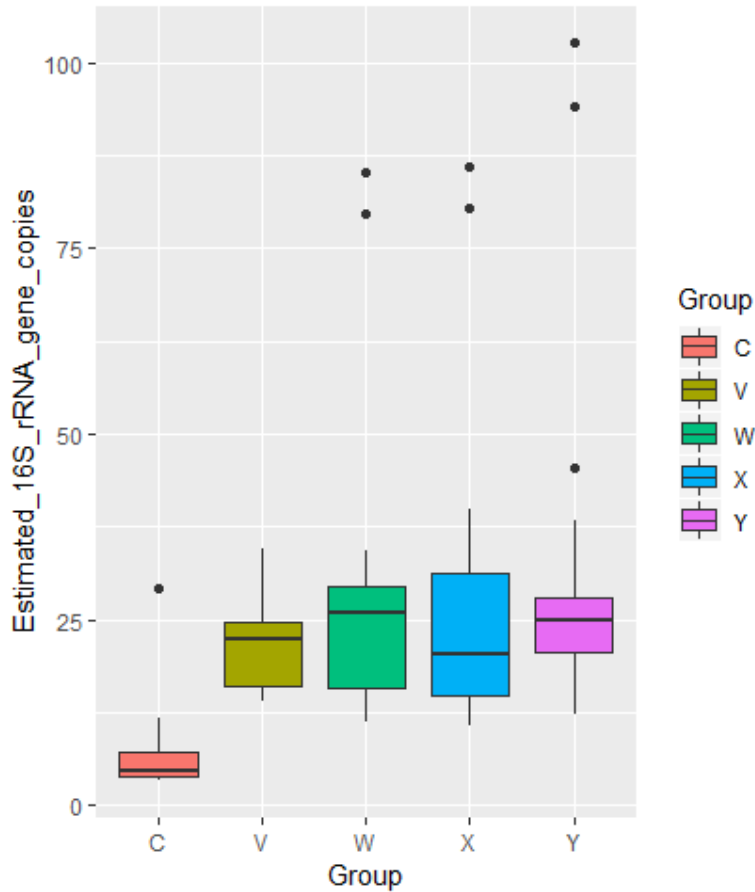


Figure 3: Distribution of estimated 16S rRNA gene copies/ μ l of blood, determined by qPCR, represented in Box-plot charts. C: negative control samples, V: young control cohort, W: elderly healthy cohort, X: AD group, Y: healthy co-habitants group. High value outliers in group Y (380.71 copies/ μ l) has been excluded from the graph.

Table 7: Summary of 16S rRNA copy number per μ l of blood, in different study groups

Study group	Average (copies/ μ l blood)	Standard deviation
Negative controls (C)	7.049	6.613
Young control (V)	21.563	20.859
Old control (W)	28.278	22.185
Co-habitants (Y)	51.384	20.010
Alzheimer's (X)	27.638	5.575

The estimated 16S rRNA gene copy numbers in the control samples were significantly lower than in the other groups. When the four study groups were compared, ANOVA showed no significant difference between the groups (p-value 0.162). However, when the negative controls group (C) was included in the analysis, a significant difference was

observed (p-value 0.0312). This indicates that the bacterial DNA obtained from the 79 participants was most likely isolated from the blood itself, and contaminants such as skin bacteria, or other microbial DNA potentially present in the environment, were not a confounding factor. These results further indicated that the concentration of bacterial DNA in the blood of AD patients is not significantly different from that of their co-habitants or from the blood of other independent old and young healthy individuals.

4.3.3 16S rRNA gene sequencing

Next generation sequencing of the 16S rRNA gene was performed using the Illumina MiSeq platform to investigate the putative bacteria present in human blood of AD patients and healthy individuals. Before DNA library preparation, PCR was performed using the HotStartTaq Plus Master Mix kit (Qiagen) on all 79 human samples, and 15 negative controls (NTCs). This was followed by agarose (2% (v/v)) gel electrophoresis. Amplification of all samples was successful, and nine out of the 15 NTC samples also showed amplification. Those nine NTCs, together with the 79 samples, were used for library preparation and pair-end sequencing of the hypervariable regions V3-V4 in the MiSeq platform.

The raw sequences from different MiSeq runs were combined and analysed in QIIME II (Caporaso *et al.*, 2010). The sequences were filtered and demultiplexed according to their respective barcodes. Sequence read length ranged from 358 to 568 base pairs (bps). The reads were further analysed by DADA2 (CALLAHAN *et al.* 2016). Barcodes and primer sequences were removed and sequence length was standardised to 358 bps. All sequencing reads chosen had a Phred quality score not lower than 23. An OTU table and representative sequences table were then constructed from the filtered sequences summarised in the table below (Table 8).

Table 8: Summary of filtered sequences after DADA2 analysis

Statistic	Number of sequencing reads
Maximum	1 065 139
Minimum	7 478
Mean	87 675
Median	56 779
Total	7 715 382

A midpoint rooted tree was constructed after MAFFT alignment. Taxonomy classification for all OTUs was then obtained using the SILVA data base for 16S rRNA data (97% classifier) (QUAST *et al.* 2012). All OTUs present in the nine NTC samples were removed from the OTU and taxonomy table. Originally, a total number of 4429 OTUs were present, after filtering those present in the NTCs samples 3790 remained. The data was imported into the phyloseq R package (MCMURDIE AND HOLMES 2013), for further processing. From the phyloseq object, all OTUs that were unassigned or unknown at the kingdom and phylum level (1456) were removed. No taxa corresponding to mitochondria or chloroplasts were present in the phyloseq object.

Only four Archaea OTUs were present in the data set, and they were also removed. All singletons were deleted from the data sets. Then, the number of sequencing reads per sample was rarefied. In order not to lose many samples, rarefaction was done at 645. Even though the standardisation of data by rarefaction has become an open discussion (MCMURDIE AND HOLMES 2014), this is the standard and still widely accepted method to normalise sequencing reads per sample. A rarefaction curve was constructed to visually confirm the inclusion of most OTUs to avoid misrepresentation of diversity due to a poor sampling strategy (Figure 4).

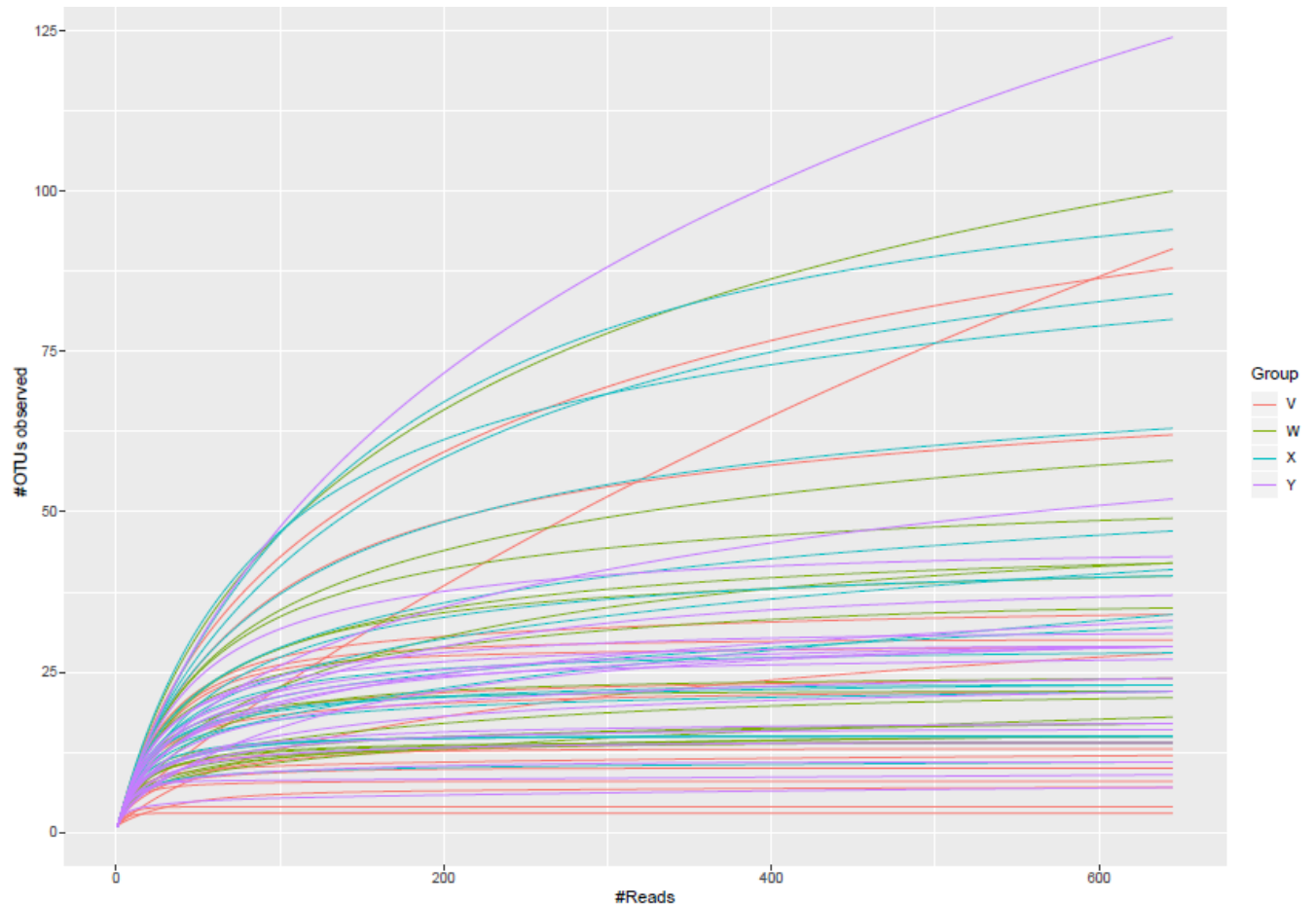


Figure 4. Rarefaction curve showing the sampling depth when 79 samples were rarefied at 645 reads per sample. Most samples have reached a plateau, indicating that sampling depth at that specific length will give a good representation of unique OTUs found in each sample.

When rarefaction was performed at 645 sequencing reads per sample, eight samples were lost, three from the AD group (X), three from the young healthy group (V), and two from the old healthy control cohort (W). When sequencing depth (number of reads) was plotted against OTUs observed, we concluded that the chosen rarefaction value was adequate. Even though curves for certain samples could have had more unique OTUs, most samples had reached, or were almost in a plateau state, indicating that most OTUs have been included in the analysis, even after rarefaction.

For further analysis the samples were analysed all together, or were grouped, depending on the aim of the particular analysis performed. When study groups were compared in pairs, one or more sample was randomly discarded in order to have the same number of

samples per group. When the “pre-treatment” of the sequences was finalised we proceeded to analyse the composition and diversity of the putative blood bacteria, based on their DNA.

4.3.4 Bacterial community composition and diversity analysis

4.3.4.1 Blood bacteria taxonomy

Sequencing of the 16S rRNA gene allowed us to investigate the taxonomy of the putative blood bacteria present in AD and healthy individuals. After sequencing, SILVA database 97% classifier was used to infer taxonomy from 16S rRNA sequencing reads (QUAST *et al.* 2012). First, possible significant differences between the four different groups' taxonomy was investigated. No significant difference was observed at any taxonomic level when Kruskal-Wallis one-way analysis of variance was performed. At phylum level, a similar trend across the four groups was observed (Figure 5). Proteobacteria was the most dominant phylum, followed by Firmicutes, Actinobacteria and Bacteroidetes. The most abundant classes of these four phyla were also plotted as relative abundance graphs (Figure 6).

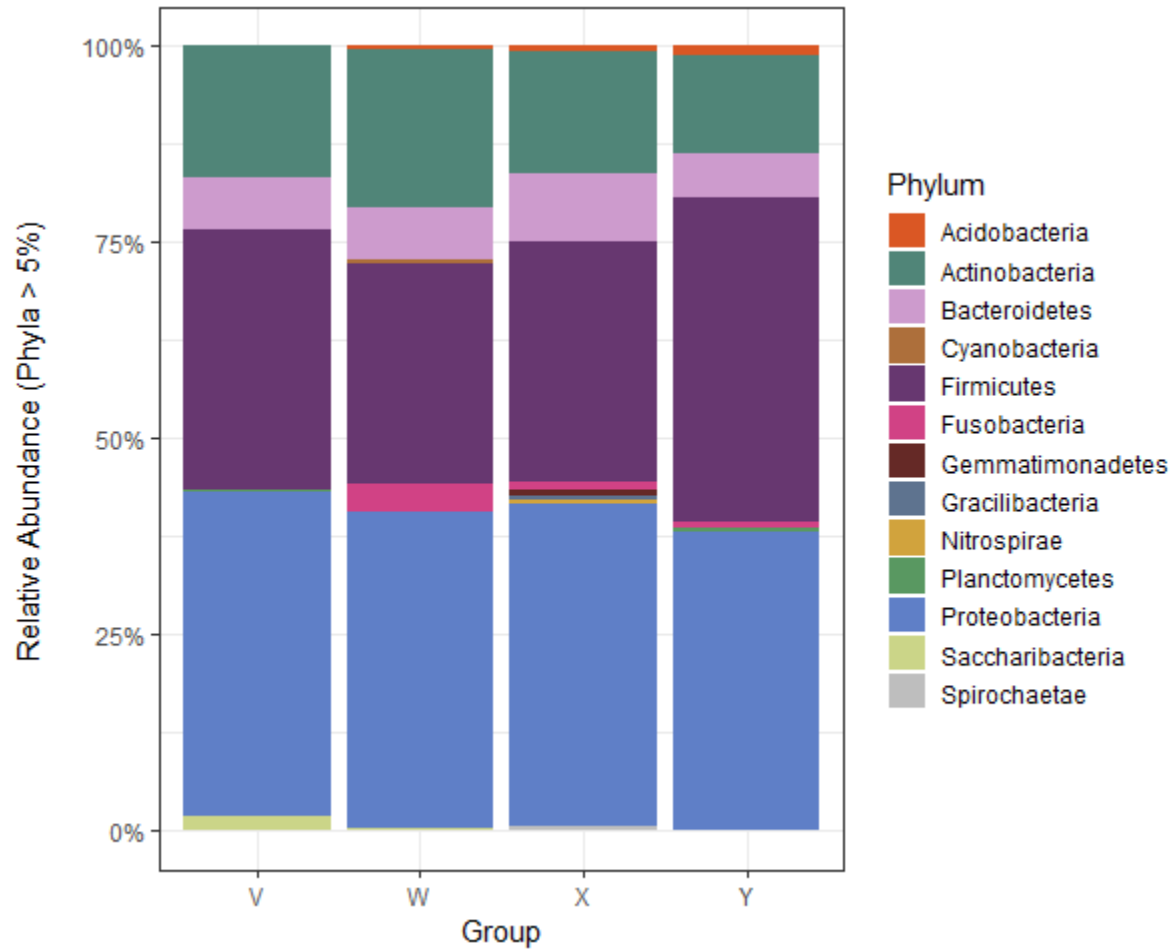


Figure 5. Relative abundances of phyla identified in the blood of all study participants, at phyla level. Only phyla present at a rate greater than 5% are shown. The four groups show a similar trend. There was no significant difference between the four groups (Kruskal-Wallis, p -value > 0.05). The most abundant taxa are: Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. V, young healthy individuals; W, elderly healthy group; X, AD cohort; Y, AD cohabitant healthy group.

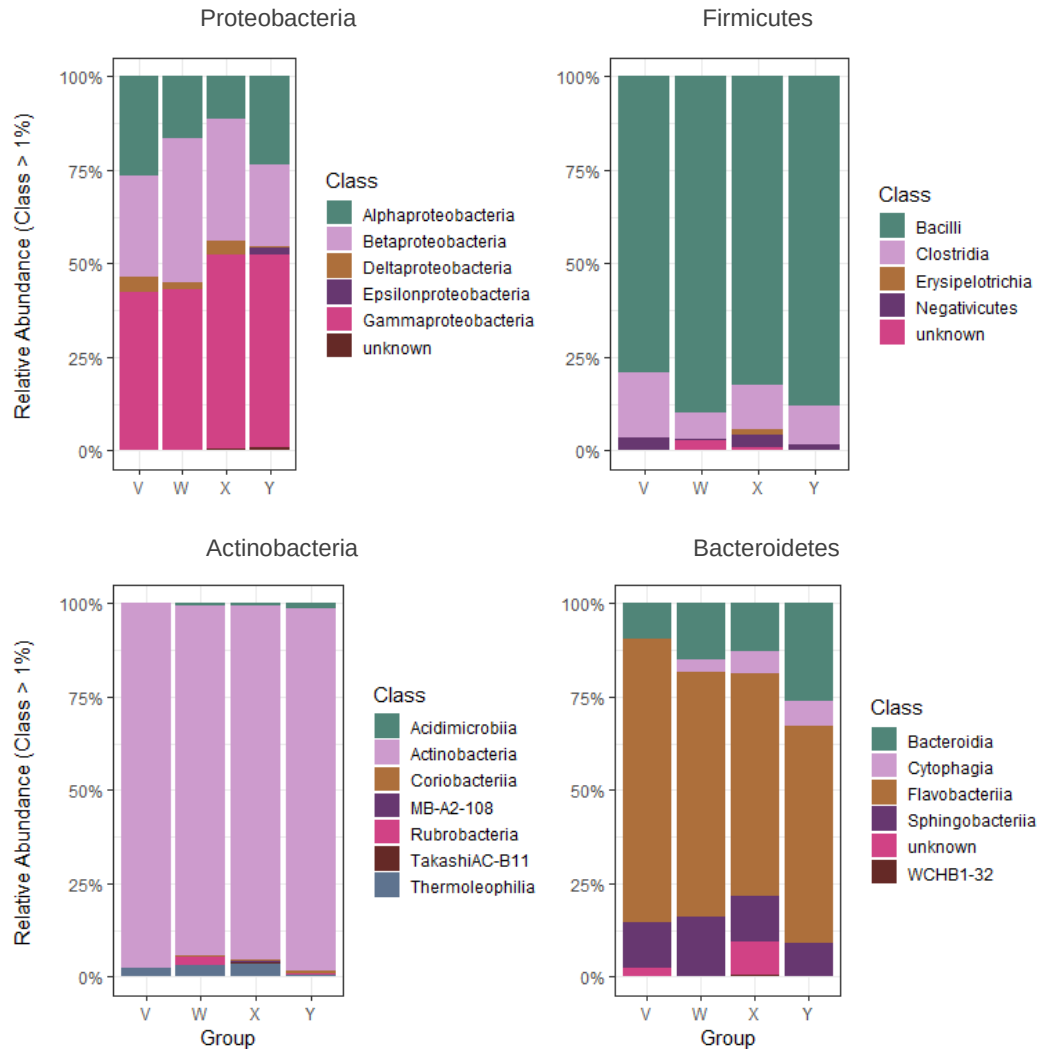


Figure 6. Relative abundances of bacterial classes belonging to the four dominant phyla found in the blood of all study participants. The patterns in different groups are similar, and no significant differences were observed in any group.

After analysing all the study groups at once, the taxonomy in the AD cohort (X) was compared with that of the healthy co-habitants group (Y). No significant differences in relative abundance was observed at any taxonomic level (Kruskal-Wallis, p -value > 0.05). Different comparisons were then performed, taking two groups at a time (X-W, X-V, W-V, W-Y, and Y-V). Once again, no significant differences between any of the groups were observed at any taxonomic level (p value > 0.05). A similar result was recorded when using the DeSeq2 algorithm (Love *et al.* 2014).

Possible taxonomical difference between the different classes of Fe levels, full blood counts and differential blood cell counts, were also tested. Relative abundance of Bacteroidetes was significantly different when red blood cell counts categories were tested (Kruskal-Wallis, p-value < 0.05) (Figure 7).

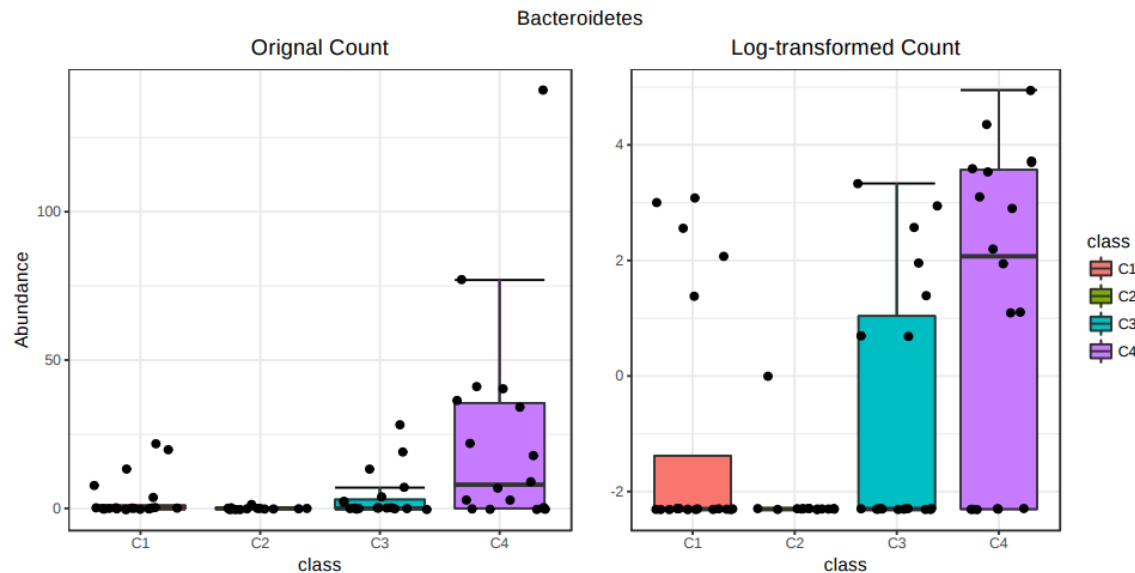


Figure 7. Relative abundance of Bacteroidetes for the four different red cell count classes. C1: $> 4.65 \times 10^9/l$, C2: $4.65 - 4.88 \times 10^9/l$, C3: $4.89 - 5.24 \times 10^9/l$, C4: $> 5.25 \times 10^9/l$. The original count of OTUs belonging to Bacteroidetes was plotted (left panel); transformed with $\log_{10}$ data (right panel).

At the class level, Flavobacteria abundance increased in individuals with higher red blood cell counts, especially C4 ($> 5.25 \times 10^9/l$), although this finding was not found to be statistically significant. On the other hand, significant differences in the relative abundance of Gammaproteobacteria was observed when the four different red blood cell classes were compared (Figure 8). No significant differences were observed at lower taxonomic levels.

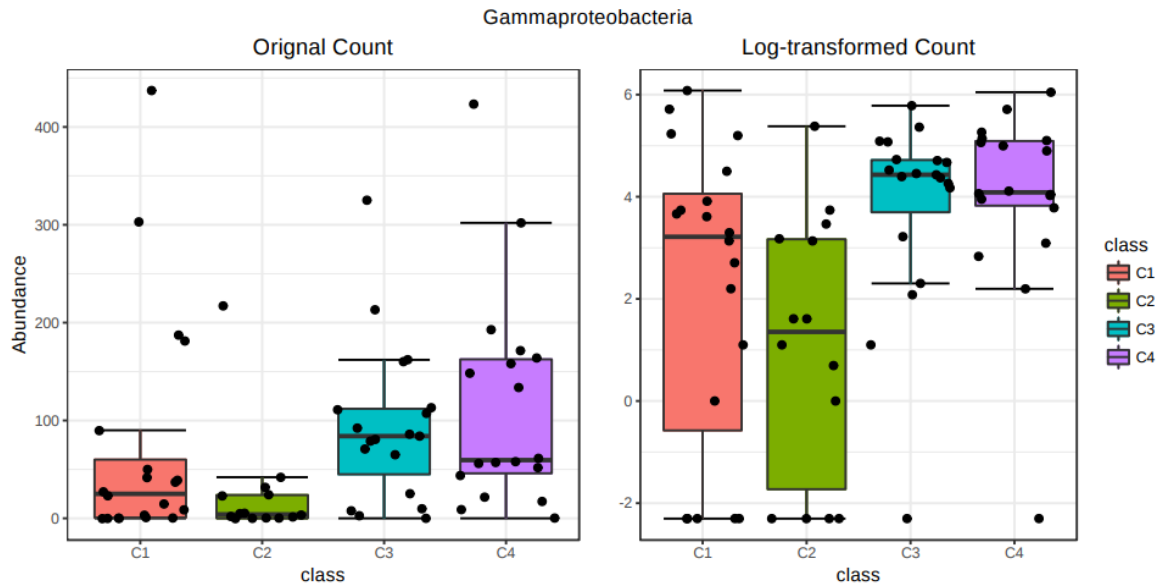


Figure 8. Original count (left) and log-transformed count (right) of Gammaproteobacteria in the blood of different groups, defined by their red blood cell count (C1-C4). C1: $> 4.65 \times 10^9/l$, C2: $4.65 - 4.88 \times 10^9/l$, C3: $4.89 - 5.24 \times 10^9/l$, C4: $> 5.25 \times 10^9/l$.

After testing whether there were significant differences in taxonomy between different groups, a Venn diagram of the OTUs present in all groups was constructed (Figure 9). Only 15 OTUs were shared between the four study groups. The great majority of OTUs identified in the present study were group specific. Only 9.94% of OTUs were shared between any two or more groups. Then, it was investigated which group-unique OTUs were present in more than one sample, within each group (*i.e.* the most frequent group-unique OTUs were identified). Four OTUs were present in three samples from the AD group, while only one OTU was shared in three samples from the healthy elderly control group. When considering the co-habitant- and young control groups, no OTUs were shared by more than two samples per group. Ten OTUs were shared by two samples within the co-habitants group, and nine by two samples belonging to the young cohort. The high percentage of group-unique OTUs, and low frequency of OTUs shared per sample within the AD group, indicates that each sample had a very unique OTU composition.

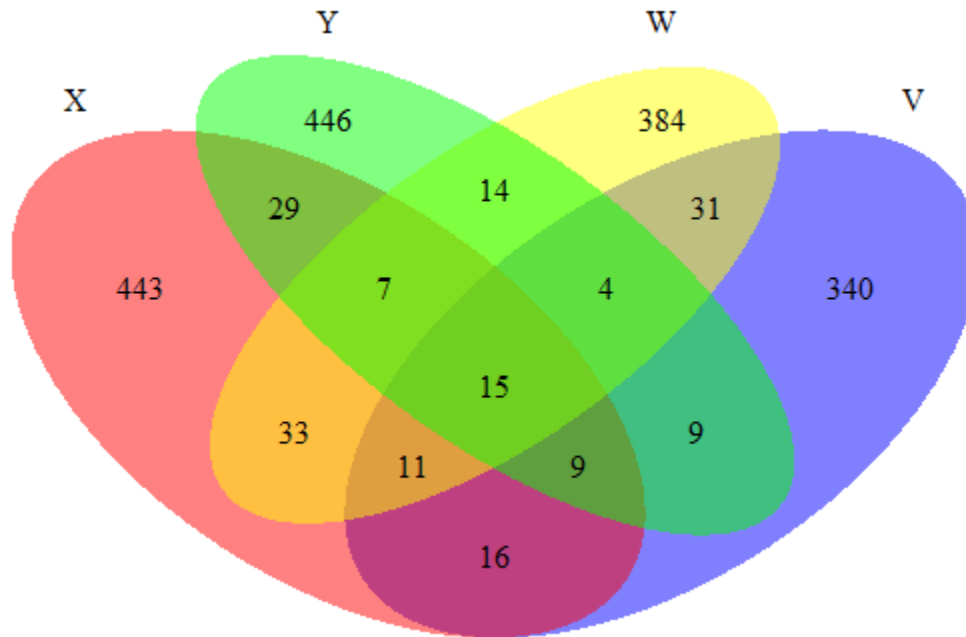


Figure 9. Venn diagram of OTUs identified in different study groups and shared between them: AD group (X), healthy co-habitants (Y), elderly healthy control group (W), and young healthy control group (V). Most of OTUs (90.06%) were not shared between any groups (*i.e.* group specific). Only 15 OTUs were shared between the four study groups.

Comparisons between unique OTUs observed in AD and healthy individuals were also carried out. The three healthy groups (V, W and Y) were grouped together, and another Venn diagram to be able to visualise the unique OTUs in each group was constructed (Figure 10). Only 120 OTUs were shared between AD patients and healthy individuals, as most of them were unique to each group. Within the 443 unique OTUs observed in the AD group, four were shared among three samples; on the other hand, four OTUs were shared between four samples in the healthy group (Table 9).

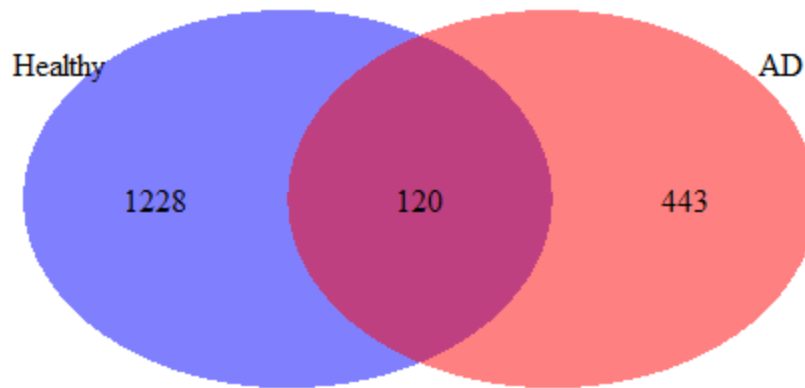


Figure 10. Venn diagram of OTUs present in all healthy individuals and in the AD study group. Only 120 (6.7%) were shared between healthy and AD individuals. The greater number of healthy OTUs is explained by the greater sample size.

Depth in taxonomical classification of the mentioned OTUs varied, therefore, the identification of the genus of only three OTUs could be achieved. The taxonomy of two OTUs found in the two different groups (AD and Healthy) appeared to be the same. However, those OTUs were categorised as unique to their own group as they could not be classified at the species level, and their OTU ID differed.

Table 9. Taxonomy of most frequent OTUs in AD and healthy individuals

Phylum	Class	Taxonomic levels			Group
		Order	Family	Genus	
Proteobacteria	Alphaproteobacteria	Sphingomonadales	NA	NA	AD
Proteobacteria	Alphaproteobacteria	Rhizobiales	NA	NA	AD
Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Moraxellaceae</i>	<i>Acinetobacter</i>	AD
Actinobacteria	Actinobacteria	Micrococcales	NA	NA	AD
Proteobacteria	Alphaproteobacteria	Sphingomonadales	<i>Sphingomonadaceae</i>	NA	Healthy
Proteobacteria	Gammaproteobacteria	Pseudomonadales	<i>Moraxellaceae</i>	<i>Acinetobacter</i>	Healthy
Bacteroidetes	Flavobacteriia	Flavobacteriales	<i>Flavobacteriaceae</i>	<i>Cloacibacterium</i>	Healthy
Actinobacteria	Actinobacteria	Corynebacteriales	<i>Corynebacteriaceae</i>	unknown	Healthy

After studying group-unique OTUs, Pearson correlation analysis was performed to investigate whether there were potential relationships between taxa abundance and the

parameters studied. We first performed correlation tests at phylum level. A positive correlation between the number of eosinophils present in the blood and the abundance of Saccharibacteria and Acidobacteria were identified in the healthy elderly control group. Another positive correlation observed in the caregiver group was identified to be between the number of neutrophils in the blood and Actinobacteria abundance. Interestingly, the same phyla showed a significant negative correlation with lymphocyte count in the same study group.

We were also interested in investigating if the correlations described earlier could be identified at lower taxonomic levels. The abundance in the class Actinobacteria showed the same type of correlations to neutrophils and lymphocytes, as seen for the phylum Actinobacteria. The number of eosinophils in the blood were not correlated to any class under Saccharibacteria or Acidobacteria, but a positive correlation to Deltaproteobacteria abundance was recorded in the healthy young control group (V). At the order level, Micrococcales and Bacillales abundance was positively correlated to basophil counts in the blood of participants in the caregiver group (Figure 11).

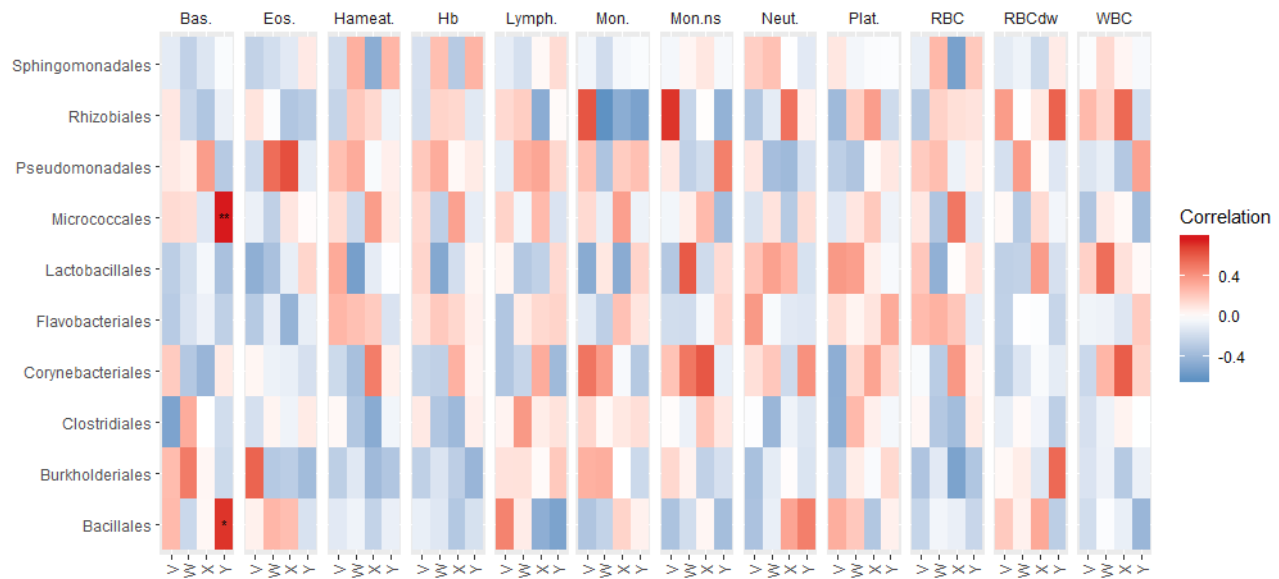


Figure 11. Heat map of Pearson's correlations between phyla abundance in the different study groups (X, Y, W and V) and blood parameters tested. Significant correlations are indicated by asterisks (p-value < 0.05 (*), >0.01 (*), >0.001 (***)). Colour corresponds to R- correlation coefficient, red colour indicates a positive correlation and blue a negative correlation. Certain blood parameters, which did not present any significant correlation, are not shown in the graph (mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration, and mean corpuscular volume). Bas., Basophil count ($\times 10^9/l$); Eos., Eosinophil count ($\times 10^9/l$); Haemat., haematocrit (l/l); Hb., haemoglobin concentration (g/dl); Lymph., lymphocyte count ($\times 10^9/l$); Mon., monocyte differential count (%); Mon.ns, monocyte total count ($\times 10^9/l$); Neut., neutrophil count ($\times 10^9/l$); Plat., platelet count($\times 10^9/l$); RBC, red blood cell count ($\times 10^9/l$); RBDdw, red blood cell distribution width (%); WBC, white blood cell count ($\times 10^9/l$).

4.3.4.2 Alpha diversity

Alpha diversity of bacteria in the blood was inferred from the sequencing data obtained and pre-processed in QIIME II (CAPORASO *et al.* 2010) and phyloseq package in R (McMURDIE AND HOLMES 2013). Alpha diversity analysis was performed for each group using microbiome-seq R package. Observed diversity, Chao1, Richness, Simpson and Shannon indices were calculated, and differences between groups were obtained using ANOVA (Figure 12).

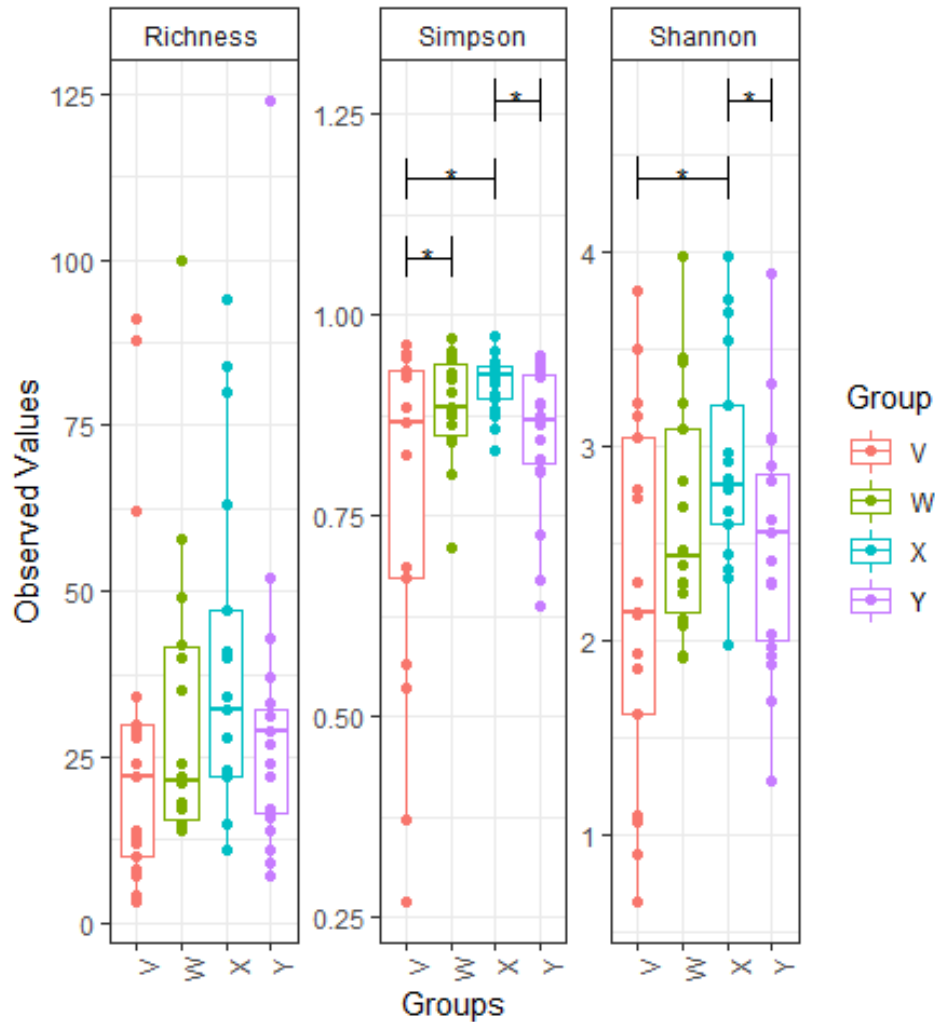


Figure 12. Alpha diversity (only showing richness, Simpson and Shannon indices) for AD group (X), co-habitants (Y), young- (V) and elderly (W) control groups. Significant differences obtained by ANOVA (p-value < 0.05) are indicated by an asterisk (*).

Observed and Chao 1 alpha diversity were not significantly different (ANOVA, p-value > 0.05) when the four different groups were compared. No significant differences were observed between the OTU richness in the blood of individuals belonging to the four different groups. Differences in Simpson diversity (ANOVA, p-value < 0.05) were observed between groups V-W, V-X, and X-Y. According to these findings, blood bacterial alpha diversity in the healthy young group (V) was lower than in the healthy elderly control group (W) and the AD group (X). This might indicate that young people have a lower bacterial alpha diversity in their blood than old people. When AD patients (group X) were compared with individuals in the caregiver group (group W), an increased alpha diversity

as a reflection of higher richness and especially evenness in blood bacterial diversity in the AD group was observed.

Differences in Shannon diversity were also observed between study groups. Significant differences (ANOVA, p -value < 0.05) were recorded when comparing the AD group with young healthy controls (V) and the AD co-habitants (Y). These findings corroborate that AD patients have a higher alpha diversity than their healthy cohabitant counterparts. It is also interesting to note that again young individuals showed a lower alpha diversity than older participants, in this case AD patients.

4.3.4.2.1 Alpha diversity and blood components

Full and differential blood count parameters, as well as serum Iron levels, were compared to blood bacterial alpha diversity of the four different groups. The aim of this analysis was to test if there was any correlation between the different blood parameters tested in this study and bacterial alpha diversity. Pearson correlations between all blood parameters and alpha diversity of different groups were performed. We only report on correlations that were significant (p -value < 0.05) (Table 10).

Table 10: Correlations between blood parameters and blood bacteria alpha diversity

Group	Blood parameter	Alpha diversity index	P-value	R coefficient
AD patients (X)	RBC distribution width (%)	Chao1	0.040	0.50
	White cell count ($\times 10^9/l$) *	Observed	0.014	0.58
	Neutrophils count ($\times 10^9/l$)	Observed	0.025	0.53
	Lymphocytes count ($\times 10^9/l$)	Observed	0.039	-0.50
Young healthy cohort (V)	Eosinophils count ($\times 10^9/l$)	Shannon	0.008	-0.62
		Simpson	0.019	-0.56
	Monocytes (%)	Shannon	0.015	-0.58
		Observed	0.001	-0.61
	Platelet count ($\times 10^9/l$)	Observed	0.026	0.54
	White cell count ($\times 10^9/l$) *	Chao1	0.043	0.50
	Red cell count ($\times 10^9/l$)	Chao1	0.044	0.49
	Mean corpuscular volume (fl)	Chao1	0.022	0.55
Healthy old groups (Y & W)	Basophils count ($\times 10^9/l$)	Simpson	0.038	-0.34

X, AD patients; Y, AD co-habitants; W, old control group; V, young control group. Present in more than one group (*)

Different blood counts were analysed by Pearson's correlation test against observed, Chao1, Shannon, and Simpson alpha diversity indices of the four different study groups. Some correlations were positive and others showed a negative correlation coefficient. Correlations between alpha diversity and blood parameters were plotted, and a linear regression line drawn, according to the respective R coefficients. Some of the regression plots are shown below (Figure 13). Healthy old control groups (Y and W) only showed a moderate negative correlation against Basophil count. White blood cell counts were positively correlated to bacterial alpha diversity in more than one group (X and V). When all groups were analysed together to test for correlations between alpha diversity and blood parameters, white blood cells showed a significant ($p\text{-value} < 0.05$) positive correlation to three types of alpha diversity indices (observed, Shannon and Chao1).

Correlations between alpha diversity and blood parameters

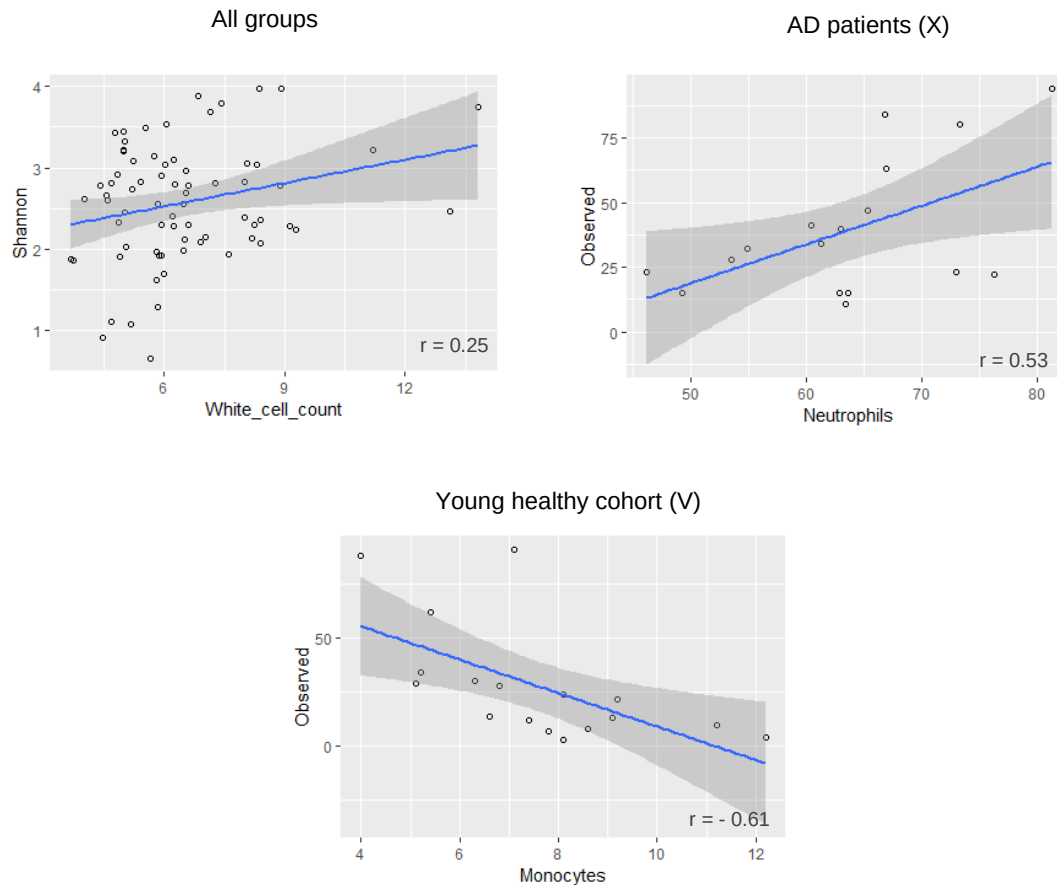


Figure 13. Examples of plots representing Pearson-correlations and regression lines calculated for alpha diversity indices of different study groups against blood parameters tested. Pearson's r-coefficient indicated for each plot at the bottom right corner. All correlations shown are significant (p-value < 0.05).

4.3.4.3 Beta diversity and community structure

After studying alpha diversity in our different study groups, and establishing certain correlations with environmental blood parameters, we investigated beta diversity and community structure, based on 16S rRNA gene sequence data. Beta diversity refers to the variation in species composition between the different groups studied. To achieve this aim, we first paired study groups in different combinations, and then studied them altogether. We constructed Principal Coordinates Analysis (PCoA) plots, based on Bray-Curtis dissimilarity, Jaccard coefficient, as well as weighted and unweighted UniFrac

distance metrics. Differences between groups were statistically tested by permutational multivariate analysis of variance (PERMANOVA).

We tested if bacterial community structure in human blood was determined by health status (AD vs healthy), age-group, sex, APOE4 genotype, ABO blood group, Rh factor, or blood parameters. In order to include serum Fe level, as well as full- and differential blood count parameters in this analysis, we transformed these numerical continuous variables into classes. Quartiles were calculated from each data set and used to classify continuous variables into four different classes: C1, C2, C3 and C4.

First, we compared the AD group (X), with their healthy co-habitants (group Y), to determine if AD patients presented a different bacterial community structure. Community structure was not defined by suffering from AD or being healthy (PERMANOVA, p-value > 0.05). Interestingly, using weighted and unweighted UniFrac, we observed a significant difference between groups belonging to different total monocyte count categories (PERMANOVA, p-value < 0.05). However, total monocyte count categories presented an R-squared value of 0.1157, which indicates that only 11.57% of the variation in distances can be explained by the number of monocytes present in the blood (Figure 14).

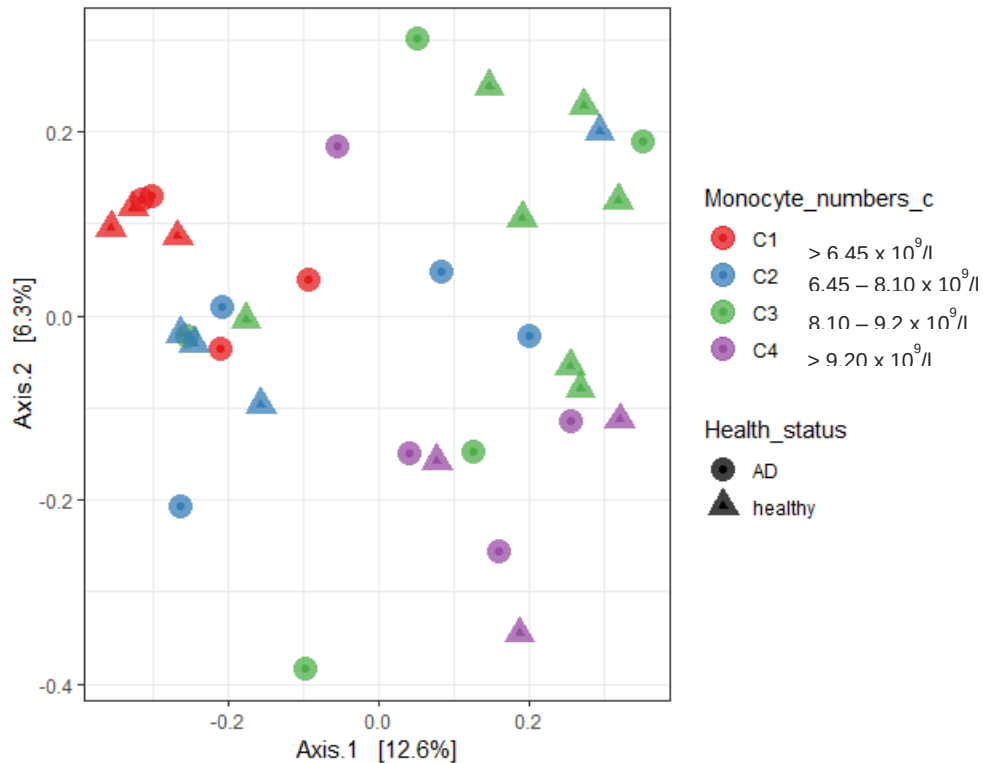


Figure 14. Bacterial community structure of AD and co-habitants. PCoA plot based on unweighted UniFrac metrics of AD group (X), and healthy co-habitants (group Y). PERMANOVA p-value < 0.05 . R-squared value 0.1157. AD patients are indicated by a circle and healthy co-habitants by a triangle. Different colours represent the four categories of monocyte numbers.

Beta-diversity in AD patients (group X) was also compared against an independent (non-cohabitant) healthy old control group (W). There were no differences in bacterial community structure between the two groups. Nonetheless, red blood cell total count (R-squared = 0.1327), haemoglobin concentration (R-squared = 0.1410), and haematocrit categories (R-squared = 0.1365) seemed to play a role in community structure when groups X and W were studied together, based on weighted UniFrac distances (PERMANOVA, p-value < 0.05 for the three variables). Even though there were significant differences, R-squared values were low, therefore clear distinct clusters could not be observed when PCoA plots were constructed. The figure below shows the ordination plot for red blood cell count, as an example (Figure 15).

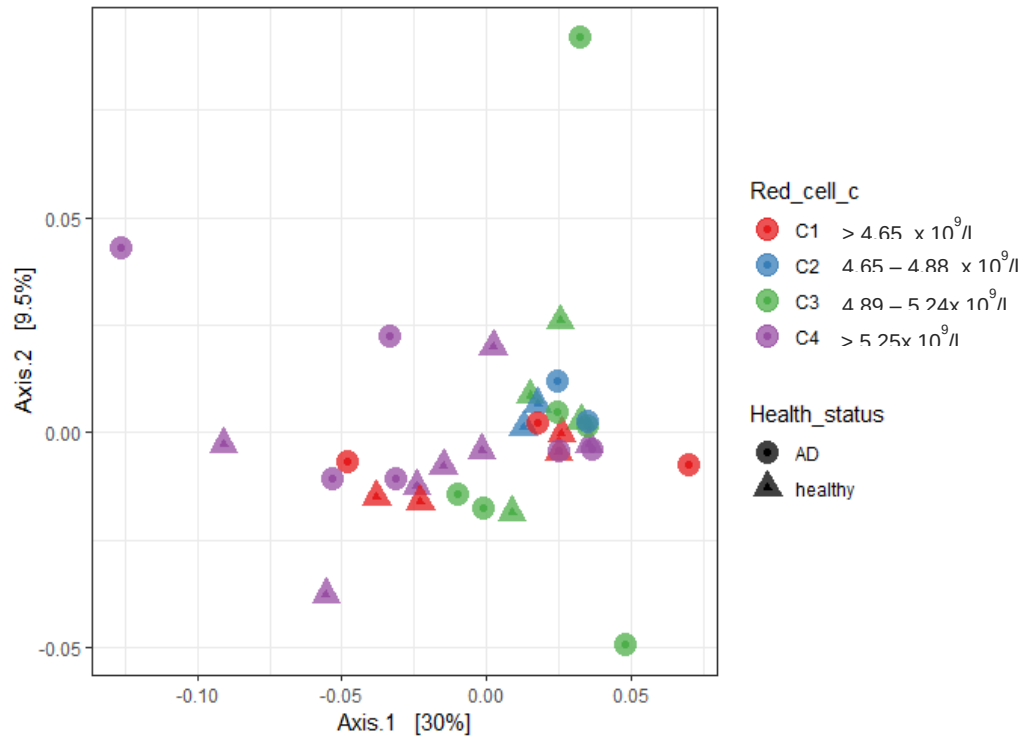


Figure 15. Bacterial community structure of AD and old healthy individuals. PCoA plot based on UniFrac distances of AD group (X), and healthy old control cohort (group Y). PERMANOVA p-value < 0.05 . R-squared value 0.1327, indicating that red blood cell count category explains 13.2% of differences. AD patients are indicated by a circle and healthy old group by a triangle. Different colours represent the red blood cell categories, based on quartiles.

We also studied the bacterial beta diversity in the blood of healthy individuals. For this purpose, we first we compared the young healthy cohort (Y) to the old healthy group W. No specific clustering pattern was observed when we looked for differences in young against elderly participants. However, when these two groups were compared using weighted and unweighted UniFrac distances, differences based on white blood cell total count (R-squared 0.1051), red blood cell total count (R-squared 0.1381), monocyte differential count (R-squared 0.1302), and monocyte total count categories (R-squared 0.1452), were significant (PERMANOVA, p-value < 0.05).

All healthy groups (Y, W and V) were analysed together in order to test if bacterial community structure was affected by sex, as well as other blood parameters tested. Sex did not influence beta-diversity. Nonetheless, based on weighted and unweighted UniFrac distances, we determined that red blood cell total count (R-squared 0.0917) and

monocyte total count (R-squared 0.0915) affected bacterial community structure (PERMANOVA, p-value < 0.05).

Finally, all groups were analysed together, and all variables were tested again to determine if they would affect bacterial community structure in the blood of all participants. The only parameter that seemed to have an effect on community structure was red blood cell count (PERMANOVA, p-value 0.0261). Based on both weighed and unweighed UniFrac distances, a PCoA plot was constructed (Figure 16). Distinct clusters were difficult to observe due to overlapping of points, thus we drew 95% confidence ellipses around each cluster. The R-squared value obtained from PERMANOVA analysis was low (0.0741), indicating that the four different red blood cell count categories only explain 7.41% of differences observed.

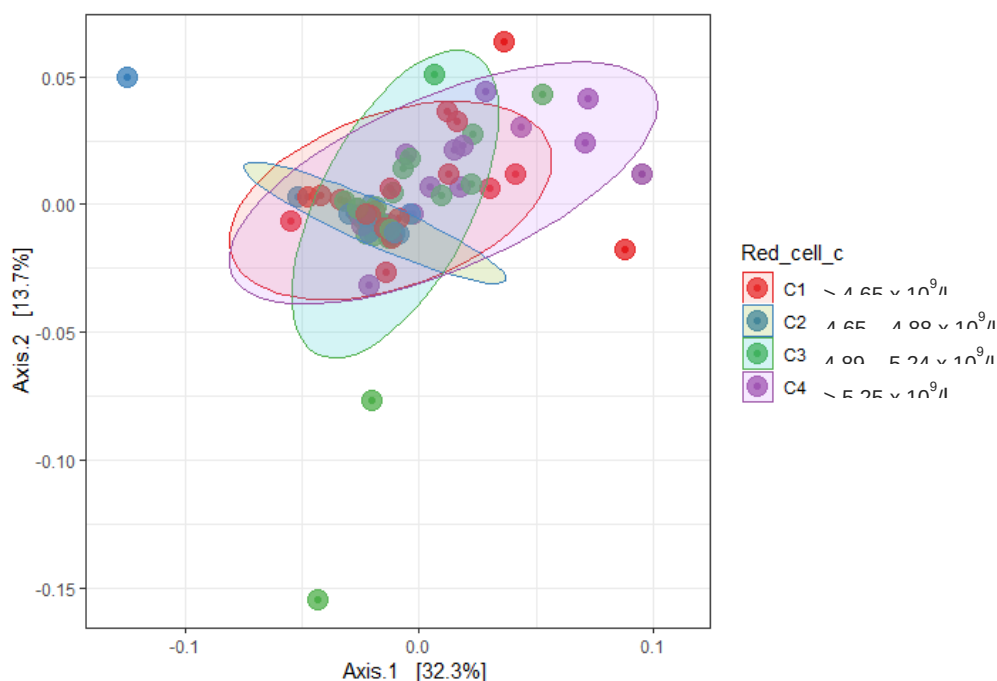


Figure 16. Bacterial community structure according to red cell counts. PCoA plot based on weighted UniFrac distances, representing bacterial community structure from blood of participants from all study groups. Different colours indicate the four distinct categories of red blood cell count, based on quartiles. The differences were tested by PERMANOVA (p-value < 0.05), R-squared values was 0.0714. Ellipses were drawn around clusters at 95% confidence level.

5. Discussion

5.1 Iron levels and blood cell counts

Haematological parameters, Fe serum level, as well as full- and differential blood counts, were obtained for all participants in the study, with the exception of five individuals, due to insufficient blood volume collected. The parameters were analysed statistically (ANOVA, Tuckey's test), to determine if significant differences existed between the four study groups: Alzheimer's disease (AD) patients, AD co-habitants, healthy elderly participants, and healthy young individuals.

Young healthy controls showed higher haemoglobin concentration (g/dl) than AD patients. Rather than being an AD specific feature, haemoglobin concentration decrease seems to be a common finding in the elderly (NILSSON-EHLE *et al.* 2000). This phenomenon is mainly due to the higher risk of developing anaemia as age increases (SMITH 2009). The other two groups composed of old participants did not show a significant decrease in haemoglobin concentration when compared to young participants, however this can be explained by dealing with a small sample size (approximately 20 individuals per group), which might not have been big enough to show the expected trend. Young individuals showed a lower red blood cell distribution width than AD patients and AD-cohabitants (both groups comprised of old individuals). This could also be explained by an increased risk of developing anaemia in the elderly, as high red blood cell distribution width is usually an indicator of different types of anaemia (EVANS AND JEHL 1991; PATEL *et al.* 2009).

An increased number of lymphocytes in the young control group was observed, when compared with AD individuals, and their co-habitants. This could be explained by the fact that lymphocyte counts are expected to be lower in the elderly, due to lower cellular immune response and lymphocyte proliferative activity (TAVARES *et al.* 2014). On the other hand, neutrophil counts were higher in AD patients when compared with young controls. No significant difference was observed in neutrophil counts between young healthy individuals and healthy elderly individuals. Since inflammation plays such an important role in the AD pathogenesis (KUYUMCU *et al.* 2012), movement of neutrophils to

the site of inflammation through the bloodstream (HUBER *et al.* 1991) could explain the elevated neutrophil numbers observed in AD patients.

5.2 APOE genotyping

The major genetic risk factor linked to late-onset AD is the *APOE* gene (CORDER *et al.* 1993; SAUNDERS *et al.* 1993; LIU *et al.* 2013), which encodes for Apolipoprotein E. This protein is involved in the metabolism of cholesterol and triglycerides (MAHLEY 1988). It has been shown that APOE4 homozygotes have an eight fold increased risk of developing late onset AD (CORDER *et al.* 1993). On the other hand, the APOE2 allele seems to decrease the risk of developing the disease (WU AND ZHAO 2016). In our study, the genotyping of the APOE gene was successfully done for all participants, with the use of qPCR.

In 2015, Luckhoff *et al.* (2015) reported on the prevalence of the APOE4 genotype in a South African population. A total number of 537 participants were part of the study. They confirmed the link between APOE4 and AD in this South African population. (LUCKHOFF *et al.* 2015). In the present study, where 80 were genotyped, a relationship of dependence between AD and the APOE4 allele, as well as the homozygote genotype E4/E4, was confirmed. When we compare the prevalence of the three different alleles in our study population against the worldwide meta-analysis study by Alzforum (ALZGENE 2010), similar trends were observed. According to the worldwide meta-analysis the prevalence of APOE – E2, E3 and E4 alleles are: 4, 58 and 38 percent, respectively in AD patients; while in this study the three alleles' prevalence corresponded to 5, 56 and 39 percent, in the same order.

As mentioned previously, APOE2 has been established as a “protective allele” against Alzheimer's dementia, especially due to its involvement in amyloid-beta clearance (WU AND ZHAO 2016). In the present study, no correlation was found between the APOE2 allele and decreasing the risk of developing AD. Considering that APOE2 is generally present in less than 10% of the population worldwide (ALZGENE 2010), a large sample size would be required to observe its effect in a specific population. It is of interest to note that the heterozygote genotype APOE E4/E2 was encountered only in healthy individuals in the

present study. Attention should be given to the interaction between APOE2 and -4 alleles, in future studies.

5.3 Bacterial DNA in the blood of AD patients and healthy individuals

According to the AD pathogen hypothesis, certain microorganisms could be involved in the pathogenesis and development of the disease (BALIN *et al.* 1998; LITTLE *et al.* 2004; MIKLOSSY 2011b; MAWANDA AND WALLACE 2013). A possible link between the presence of blood bacteria and AD was one of the hypotheses of this study. First, bacterial DNA concentration in the blood of healthy individuals (young controls, elderly controls, and AD co-habitants) was investigated by the amplification of the 16S rRNA gene, using qPCR. The gene copy numbers per microlitre of blood was then estimated, and compared between groups.

Blood microbiome dysbiosis has been reported in a number of diseases (AMAR *et al.* 2011; AMAR *et al.* 2013; DINAKARAN *et al.* 2014; SATO *et al.* 2014; MANGUL *et al.* 2016; TRAYKOVA *et al.* 2017; LI *et al.* 2018). An elevated bacterial DNA load in the blood of diseased subjects were observed in the blood of cirrhotic- (TRAYKOVA *et al.* 2017), cardiovascular disease (DINAKARAN *et al.* 2014), and diabetes patients (AMAR *et al.* 2011). However, no significant differences existed between bacterial DNA load in the blood of Alzheimer's patients when compared with their co-habitants, a healthy elderly group and a healthy young cohort, in the present study. This indicates that there is no difference between the quantity of bacterial DNA, and potentially bacterial cells, present in the blood of Alzheimer's disease when compared to healthy people; as well as when we compare young and elderly individuals.

Although no differences in 16S rRNA gene copy numbers were observed, an important finding was derived from the quantification of this gene. In studies dealing with low biomass, such as blood microbiome related research, the use of appropriate stringent negative controls is required (SALTER *et al.* 2014). Much research on novel human microbiomes has been discredited by negligence regarding the use of negative controls, for example the controversy around the placenta microbiome (LAUDER *et al.* 2016). For this reason, 15 specific negative controls were used in this study to account for potential contamination from the human skin, needles, laboratory environment, reagents and

consumables. When we compared the 16S rRNA gene copy numbers between all groups and negative controls, we observed that the estimated bacterial DNA in the four study groups were significantly higher than that detected in the negative controls, ruling out possible contamination from environments different from the blood itself. This is an important finding, as there is controversy around the existence of a blood microbiome even after important recent findings (McLAUGHLIN *et al.* 2002; MITCHELL *et al.* 2016; PAÏSSÉ *et al.* 2016). Even though DNA is used as proxy to assume the existence of bacteria in the blood of healthy and diseased people, there is evidence that supports these assumptions. For example the finding of bacterial RNA (LOOHUIS *et al.* 2018), and studies that include the use of microscopy (McLAUGHLIN *et al.* 2002; PANAIOTOV *et al.* 2018).

5.4 Bacterial community composition and diversity analysis

Bacterial DNA isolated from all study participants was studied by sequencing the 16S rRNA gene using Next Generation amplicon sequencing. Once the data was analysed, the taxonomy of the putative blood bacteria in the four study groups (Alzheimer's patients, AD cohabitants, old healthy and young healthy individuals) was obtained. The analysis allowed the resolution of taxonomy mostly at phylum, class and order levels. Lower taxonomic classification (i.e. genus and species levels) was challenging. Even though other studies also included the sequencing of only specific regions of the 16S rRNA gene (e.g. hypervariable regions V3 and V4) (GOSIEWSKI *et al.* 2016; PAÏSSÉ *et al.* 2016), our data supports the need to include sequencing of the full-length gene in future research on the blood microbiome.

The taxonomy of the AD group was compared to that found in all healthy individuals in the present study. No significant differences was observed at any taxonomic level. When sex, age-group, APOE genotypes, ABO group and Rh-factor were investigated, no taxonomic differences between groups were observed either. The most dominant phyla in all groups were Proteobacteria, followed by Firmicutes, Actinobacteria and Bacteroidetes. This is consistent with other studies that have investigated the blood microbiome (AMAR *et al.* 2011; DINAKARAN *et al.* 2014; PAÏSSÉ *et al.* 2016; LOOHUIS *et al.* 2018). It is important to note that the dominant bacterial phyla in the blood differed from that in the skin and other body tissues such as the gut, the oral cavity and the urogenital

tract (D'ARGENIO AND SALVATORE 2015). The dominant phyla in the gut are Firmicutes and Bacteroidetes (QIN *et al.* 2010a; BÄCKHED *et al.* 2012; LE CHATELIER *et al.* 2013), while the most common bacterial phylum in the skin is Actinobacteria (COGEN *et al.* 2008; GRICE *et al.* 2009). These finding further highlights the importance of sequencing the full length 16S rRNA gene in a larger study population to further investigate the potential influence of sex, age-group, APOE genotypes, ABO group and Rh-factor on blood microbiome taxonomy.

Blood parameters were transformed into categorical values to detect possible relationships between blood environment parameters and blood bacterial taxonomy. Relative abundance of *Gammaproteobacteria* in the blood seemed to be linked to red blood cells (RBC). Higher red blood cell classes ($4.89 - 5.24 \times 10^9/l$, and $> 5.25 \times 10^9/l$) presented significantly higher *Gammaproteobacteria* abundance. This bacterial class consists of a diverse group of gram-negative bacteria, including a number of human pathogens, such as some members of the genus *Pseudomonas* (WILLIAMS *et al.* 2010). The reason for a possible relationship between *Gammaproteobacteria* and RBCs needs to be further explored, especially because this correlation was not seen at lower taxonomic levels within this class. Nonetheless, it is important to mention that certain bacteria can make use of RBCs to survive by residing inside these cells (YAMAGUCHI *et al.* 2013; VITRY *et al.* 2014). An example of this is the pathogen *Francisella tularensis*, causative agent of tularaemia, which has been shown to invade and persists in erythrocytes (HORZEMPA *et al.* 2011). This bacterial species belongs to the class *Gammaproteobacteria*. It would be of interest for future investigations to perform Transmission Electron Microscopy to determine whether this association between higher red blood cell levels and higher *Gammaproteobacteria* is intracellular.

Operational taxonomy units (OTUs), which were unique to a specific group or shared between the four study groups, were also investigated. Only 120 OTUs (0.07%) were found to be shared between AD patients and all healthy controls. Moreover, only 15 OTUs (0.01%) were shared between the four groups. Since most OTUs seemed to be group-unique (more than 90% were not shared between any two groups), OTUs shared between samples belonging to the same group were further investigated. Relatively few OTUs

were shared between samples of the same group. Only four OTUs shared by three samples were identified in the AD group. Furthermore, only four OTUs were shared between four samples, when all healthy groups were analysed together. This is a clear indication that the taxa present in the blood of AD patients and healthy individuals is individual-specific, rather than group-specific, as initially thought. In other words, bacteria in the blood could have a high interpersonal variability, similar to other microbiomes such as the gut, the oral cavity, and the skin (COSTELLO *et al.* 2009; URSELL *et al.* 2012). This highlights the importance of further investigation of the blood microbiome for specific application in other fields such as immune therapy and personalized medicine. It would also be of interest to determine the temporal stability of this microbiome, and its potential relationship to pathology in a patient-specific setting.

The most common or shared OTUs that could be classified at the genus level in both in AD patients and healthy individuals were *Acinetobacter* and *Sphingomonas*. Knowledge is scarce about the role of these microorganisms in the blood. Nonetheless, we hypothesise that these OTUs could be linked to human health and disease. Members of the genus *Acinetobacter*, such as *A. baumannii*, can be an opportunistic pathogen, many times involved in hospital acquired infections to the respiratory system, as well as other infections including the skin and bacteraemia (PELEG *et al.* 2006; HOWARD *et al.* 2012). *Sphingomonas paucimobilis*, a species belonging to the order *Sphingomonadales*, is also an opportunistic pathogen that has been linked to septicaemia and bacteraemia (RYAN AND ADLEY 2010). Even though we do not know whether these particular species would correspond to the OTUs identified, it shows the possibility that there are species linked to the identified taxa, which could be part of the normal human microbiome and some with potential medical relevance. An urgent need exist to develop tools sensitive enough to characterize the blood microbiome at species level.

Relative abundances of blood bacteria at different taxonomy levels were also tested for significant correlations to all blood parameters used in the study. Pearson's correlation was used to study all continuous variables (Fe-, haemoglobin-, red-, and white blood cell concentrations) that represent the haematological environment, where potential blood bacteria would reside. At the order level, a positive correlation between Basophils and the

relative abundance of *Micrococcales* and *Bacilliales* was observed in the AD cohabitants old controls group. Basophils are a type of white blood cell, which play a role in allergies, as well as parasitic infections (LANTZ *et al.* 1998; FALCONE *et al.* 2000). The exact reason for an increase in Basophil numbers in the blood accompanied with an increase in *Micrococcales* and *Bacilliales* remains to be further investigated. Nevertheless, there are bacterial species belonging to those orders, which could be of relevance in human health and disease. For example, *Bacillus*, *Listeria* and *Staphylococcus* species, which belong to the order *Bacilliales*, represent well known pathogens and are also part of the normal human microbiome (DREVETS AND BRONZE 2008; BOTTONE 2010; LAGIER *et al.* 2012; WANG *et al.* 2014b; LIU *et al.* 2015; KENNEDY *et al.* 2017). Furthermore, an important species in the order *Micrococcales*, *Micrococcus luteus*, has been isolated from human skin, and even though it forms part of the normal skin flora of humans, in certain cases it could also cause bacteraemia (KLOOS AND MUSSELWHITE 1975; PECES *et al.* 1997).

Alpha diversity of the four study groups (AD patients, AD co-habitants, elderly and young healthy controls) was also investigated in this study. Observed, Chao1, richness, Simpson and Shannon diversity indices were calculated for each study group. Only when Simpson and Shannon diversity indices were used, significant differences were observed between study groups. These two indices take both richness and evenness into consideration (HAEGEMAN *et al.* 2013). Species richness weighs more in the Shannon index, while the Simpson index puts a greater weight on species evenness (KIM *et al.* 2017).

Alzheimer's patients showed a higher Simpson diversity than healthy young participants. However when two independent healthy groups were compared (i.e. healthy young and elderly control groups), older individuals also showed a higher diversity than young participants, indicating that the differences observed in Simpson diversity could be due to age group, rather than health status (having AD or being healthy). Nevertheless, Simpson and Shannon diversity indices for the young control group were not significantly different from that observed for the AD co-habitant groups, which also represents an elderly cohort. Moreover, when AD patients were compared to their healthy co-habitants (same age group), the co-habitant group showed a significantly lower Simpson and

Shannon diversity. This led to the conclusion that AD could be the main factor leading to an increase in alpha diversity in the study participants. A clear explanation for this finding remains to be explored. AD is characterised by an upregulation of the immune system response, to account for neuro- and systemic inflammation, which are hallmarks of the disease (PERRY 2004; EIKELENBOOM *et al.* 2006; HOLMES *et al.* 2009; HENEKA *et al.* 2015). Therefore, we can hypothesise that this phenomenon could partly explain an increase in alpha diversity, if the immune system targets the more dominant species in the blood, translating into an elevated bacterial richness and evenness.

In order to further understand differences in alpha diversity between study groups, Pearson's correlations to the haematological parameters were performed. When the four participant groups were studied together a positive correlation was observed between all alpha diversity indices and total white blood cell counts. This finding is aligned with our hypothesis that dominant bacterial species would be targeted by a more active immune system, possibly making niches available for other less abundant species, thus increasing alpha diversity as bacterial richness and evenness increase (HAEGEMAN *et al.* 2013). Even though gut bacteria inhabit an environment that is very different from the blood, knowledge about their interaction with the immune system can provide valuable information about how homeostasis is maintained (HAEGEMAN *et al.* 2013). The immune system can affect the taxonomy, diversity and location of bacteria in the gut by different mechanisms, for example the excretion of antimicrobial peptides and the activation of innate and adaptive immune responses (DUERKOP *et al.* 2009; COSTELLO *et al.* 2012; HOOPER *et al.* 2012; MACPHERSON *et al.* 2012). If the immune system is able to affect bacterial diversity in the gut, it is also possible that it ensures blood bacteria homeostasis by different mechanisms, perhaps even facilitated by their physical proximity.

Alpha diversity of each individual study group was also tested for relationships with blood environment variables. Interesting findings included a positive correlation between neutrophil counts and bacterial diversity in Alzheimer's patients. As mentioned previously, the immune system response in an AD patient is dysregulated and characterised by neuroinflammation, as well as systemic inflammation (MCGEER *et al.* 1989; HENEKA *et al.* 2015). Neutrophils are part of the innate immune system and are very important in the

initial stages of infection and inflammation (AMULIC *et al.* 2012). As neutrophil numbers increase during the inflammatory response, dominant bacterial species could be targeted, and alpha diversity may increase. It is important to mention that certain bacterial species, such as *Staphylococcus aureus* (THWAITES AND GANT 2011) and *Escherichia coli* (FEXBY *et al.* 2007), could use neutrophils to survive intracellularly, while evading other immune system responses. It is possible that different species also use neutrophils in this way, causing an increase in alpha diversity.

Most white blood cells showed a positive correlation with bacterial diversity in the blood. However, a significant negative correlation was observed between monocyte counts and bacterial diversity in the young healthy cohort. Monocytes are agranular white blood cells that can differentiate into macrophages, best known to act as part of the innate immune response, although they can contribute to acquired immunity as well (TERRITO AND CLINE 1977). It has been shown that due to immunosenescence (decay of immune system responses due to normal ageing), the function of Toll-like receptors (TLR) in monocytes and macrophages is compromised (PANDA *et al.* 2009). Therefore, a possible explanation for a negative correlation between monocyte counts in the blood of young individuals and alpha bacterial diversity, could be related to the fact that monocytes in these individuals work more efficiently on regulating less dominant bacterial species.

Differences in alpha diversity could be identified between Alzheimer's patients and healthy individuals. However, beta diversity was not different between AD and healthy participants. Moreover, community structure was not defined by sex, age group, APOE genotype, ABO blood group or Rh factor. The only factors that could explain differences in samples and clustering of specific groups were monocyte counts, as well as red blood cells. Although bacterial community structure of blood bacteria was significant when samples were grouped according to similar monocytes and red blood cell counts, these parameters only explained less than 15% of the variation. Monocytes could have a potential role in regulating bacterial diversity, as explained earlier. On the other hand, red blood cells could potentially be used by certain bacteria to evade the immune system and thrive in the blood. Examples of bacteria that can reside in erythrocytes are *Streptococcus pneumoniae* (YAMAGUCHI *et al.* 2013), *Bartonella quintana* (ROLAIN *et al.* 2002), *Brucella*

melitensis (VITRY *et al.* 2014), and *Francisella tularensis* (HORZEMPA *et al.* 2011). Future research will have to be targeted at explaining the relevance of monocyte and erythrocytes in blood bacterial beta diversity. The findings of the present study highlight the need for two very important points that need urgent further investigation: (i) characterizing the blood microbiome at species level and (ii) correlation studies of detailed immune system profiles with the blood microbiome composition.

6. Conclusion and future perspectives

The research presented in this dissertation confirms the presence of bacterial DNA in blood of healthy individuals, as well as in diseased patients, and further highlights the importance of these research topics. Although controversy still surrounds the concept of a human blood microbiome, researchers currently make use of bacterial DNA and RNA, to characterise bacterial cells inhabiting this environment. In this study, the use of stringent negative controls showed that bacterial DNA isolated originated from the blood. Quantification of the 16S rRNA gene, demonstrated that the estimated number of bacterial DNA copies obtained from negative controls was significantly lower and negligible, when compared to bacterial DNA obtained from all study participants. However, no significant difference in bacterial quantity between AD patients and healthy participants were observed.

Bacterial DNA concentration in the blood of people suffering from a disease, such as diabetes, cirrhosis and cardiovascular disease, tends to be higher when compared to healthy individuals. This would suggest a possible link between blood bacteria and the particular disease. In this study, we did not observe any significant difference in the 16S rRNA gene copy number between AD patients and young and elderly healthy individuals. Although this is a possible indication that there might not be a link between blood bacteria and AD, the bacterial composition and diversity in the blood of Alzheimer's patients and healthy individuals was investigated. Moreover, the possible effects that AD risk factors and haematological parameters could have on the blood microbiome of AD and healthy individuals was also studied.

Information regarding the blood environment, where bacteria could potentially exist, was gathered. Specific differences between the different study groups, in haemoglobin concentration, red cell distribution width, lymphocytes and neutrophil counts, were found. Certain blood environment parameters were linked to blood bacterial taxonomy and diversity. We were specifically interested in studying AD risk factors that could potentially affect blood bacteria in these patients. In the population studied, a relationship of dependency was confirmed between AD and the major risk gene factor APOE4, as

expected. However, APOE4, as well as other AD risk factors such as sex, and ABO blood group, did not have any effect on bacterial taxonomy or diversity.

After next generation sequencing of the 16S rRNA gene, and thorough data analysis, we did not observe differences at any taxonomic level, when AD patients and healthy individuals were compared. Certain hematological parameters measured, such as erythrocyte counts and specific leukocytes (e.g. neutrophils and basophils) were found to play a role in the abundance of different taxa. It was further confirmed that most blood bacterial OTUs were not shared between groups, nor between individuals belonging to the same group. This gives an indication that the blood microbiome might present high interpersonal variability, similar to the skin and the gut microbiomes.

Blood bacterial diversity analysis, revealed significant differences in alpha diversity between AD patients and healthy individuals, as well as between young and elderly participants. Alzheimer's patients presented with a higher alpha diversity than healthy individuals. This could be explained by interactions between blood bacteria and the immune system. Certain correlations between white blood cells, such as neutrophils and monocytes, and bacterial alpha diversity were identified. Although we hypothesize that the AD neuro- and systemic inflammatory state could contribute to this rise of bacterial evenness and richness in the blood of these patients, further research needs to be conducted to fully elucidate the interaction between blood bacteria and the immune system. Regarding beta diversity, age, sex, APOE genotype, ABO group, Rh factor and iron serum levels, could not be linked to blood bacterial community structure. However, red blood cell- and monocyte abundance in the blood could partially explain differences in beta diversity.

Taken together the findings of this research it can be concluded that there is bacterial DNA in the blood of AD patients as well as in healthy individuals. Alzheimer's patients do not present with a higher bacterial load in their blood, when compared to healthy participants. These patients showed a higher bacterial alpha diversity than healthy individuals, but beta-diversity does not differ. AD associated risk factors do not influence bacterial taxonomy or diversity. On the other hand, certain haematological parameters,

such as red- and white blood cell counts, do influence bacterial taxonomy, as well as alpha- and beta diversity.

One of the limitations that this study presents is the sample size, especially of AD patients. Only twenty of them could be recruited for the study, turning the investigation of bacterial community structure, and differences between AD and healthy blood bacteria, rather challenging. Moreover, taxonomic resolution at species and genus levels was quite limited. We used a specific region between the V3 and V4 hypervariable regions of the 16S rRNA gene for targeted next generation amplicon sequencing. As an alternative, the whole 16S rRNA gene should be used for sequencing, to make taxonomic assignment more efficient. Finally, the use of DNA as a proxy for viable and active bacteria in a specific environment is always problematic. We are conscious of this limitation, nonetheless, previous studies on the human microbiome have shown that this technique could be effective, especially as the first step when characterising of a novel microbiome.

In future studies, the relationship between the immune system and the blood microbiome needs to be explored. Furthermore, shot-gun metagenomics sequencing should be performed in order to improve taxonomic assignment, as well as to investigate the possible function that blood bacteria potentially perform in the blood of healthy and AD individuals. Research based on bacterial RNA is also needed, to elucidate whether these bacteria are potentially active. Although most blood bacteria is believed to be unculturable, culture methods that mimics the physiological state, followed by sequencing, could be of use to assess if taxa known to be culturable are living and active in the human blood. The study of the human blood microbiome at different time points could also benefit our understanding about the potential dynamic nature of this microbiome. Finally, the relationship between bacteria present in the human blood and in other body locations such as the skin, the mouth, and the gut, needs to be explored. With this study, we have just scratched the tip of the iceberg. Blood microbiome research will uncover a fascinating new world in the study of human health and disease.

7. Bibliography

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8. Appendix

Table A1: Blood parameters of all participants

Sample	Fe	WBC	RBC	Hb	Htct.	MCV	MCHb	MCHbC	RBCdw	Plat.	Neut.	Lymph.	Mon.	Mon.ns	Eos.	Bas.
V001	26.8	5.19	5.01	15.6	0.455	90.8	31.1	34.3	12.6	133	51.0	38.2	9.2	0.48	1.2	0.2
V002	30.2	7.42	4.87	14.7	0.452	92.8	30.2	32.5	12.2	318	63.2	31.1	4.0	0.30	0.9	0.4
V003	9.9	5.52	4.80	15.3	0.445	92.7	31.9	34.4	11.8	213	50.3	40.6	6.5	0.36	2.2	0.2
V004	11.5	4.99	4.65	14.6	0.435	93.5	31.4	33.6	13.2	236	45.1	47.5	5.2	0.26	1.4	0.8
V005	11.5	5.54	5.54	17.2	0.503	90.8	31.0	34.2	12.7	249	45.3	47.3	5.4	0.30	0.9	0.7
V006	25.5	5.74	4.35	13.1	0.411	94.5	30.1	31.9	12.9	316	51.5	41.5	6.3	0.36	0.2	0.3
V007	25.6	4.69	4.73	14.5	0.428	90.5	30.7	33.9	12.6	274	45.2	39.2	6.8	0.32	7.5	0.9
V008	13.7	6.58	4.82	14.4	0.441	91.5	29.9	32.7	13.7	262	64.8	24.5	8.1	0.53	1.5	0.8
V009	22.7	3.73	5.25	15.9	0.467	89.0	30.3	34.0	11.8	186	50.4	37.0	8.6	0.32	3.2	0.5
V010	45.9	5.80	4.66	14.1	0.421	90.3	30.3	33.5	12.5	268	55.6	36.6	6.6	0.38	0.7	0.5
V011	21.2	5.66	4.71	15.3	0.452	96.0	32.5	33.8	13.4	235	46.1	43.8	7.8	0.44	1.6	0.5
V012	14.0	5.56	4.73	13.2	0.407	86.0	27.9	32.4	13.6	273	53.3	34.3	7.2	0.40	4.0	0.9
V013	19.1	6.59	5.82	17.5	0.515	88.5	30.1	34.0	12.4	215	56.2	33.1	9.1	0.60	0.5	0.6
V014	16.4	5.15	4.86	14.2	0.425	87.4	29.2	33.4	12.3	118	51.2	31.3	12.2	0.63	4.3	0.8
V015	11.2	9.79	5.34	17.0	0.510	95.5	31.8	33.3	13.6	270	48.0	39.8	7.0	0.69	4.2	0.9
V016	16.3	8.05	5.28	16.0	0.491	93.0	30.3	32.6	13.2	245	60.1	32.0	5.1	0.41	2.2	0.5
V017	14.7	8.18	5.83	17.2	0.479	82.2	29.5	35.9	12.6	293	61.7	27.3	7.1	0.58	3.2	0.5
V018	10.5	7.01	4.49	14.5	0.429	95.5	32.3	33.8	13.3	307	54.0	36.5	7.4	0.52	1.4	0.6
V019	14.6	4.47	4.80	14.9	0.447	93.1	31.0	33.3	12.4	180	39.1	41.6	8.1	0.36	10.5	0.7
V020	18.5	7.59	4.43	14.7	0.432	97.5	33.2	34.0	14.1	226	32.8	49.9	11.2	0.85	5.0	0.8
W001	17.4	6.91	4.90	15.9	0.459	93.7	32.4	34.6	12.7	254	58.6	29.2	9.4	0.65	1.9	0.6
W002	14.7	4.69	5.25	14.4	0.471	89.7	27.4	30.6	14.4	297	44.2	45.2	8.1	0.38	1.7	0.6
W003	10.6	9.27	4.90	15.2	0.462	94.3	31.0	32.9	13.7	304	56.0	34.5	5.1	0.47	3.6	0.4
W004	12.5	5.92	5.06	16.2	0.488	96.4	32.0	33.2	14.7	167	42.6	49.2	6.1	0.36	1.4	0.5

Table A1 continuation

W005	15.4	8.24	4.80	14.8	0.422	87.9	30.8	35.1	12.6	204	71.3	19.4	8.1	0.67	0.6	0.2
W006	17.0	6.11	4.27	13.5	0.403	94.4	31.6	33.5	13.6	173	69.5	21.3	5.2	0.32	2.6	1.1
W009	19.3	4.65	4.53	14.0	0.415	91.6	30.9	33.7	14.0	248	51.4	37.2	9.7	0.45	1.1	0.4
W010	11.4	8.38	5.84	17.5	0.521	89.2	30.0	33.6	13.8	169	73.7	15.6	7.0	0.59	2.7	0.4
W011	18.3	4.77	5.52	16.4	0.487	88.2	29.7	33.7	12.3	240	57.5	30.2	7.5	0.36	3.8	0.4
W012	16.5	4.97	4.99	15.7	0.462	92.6	31.5	34.0	12.9	262	34.2	58.8	5.0	0.25	1.4	0.4
W013	15.7	5.23	5.50	16.6	0.480	87.3	30.2	34.6	14.4	148	70.2	18.5	9.2	0.48	1.5	0.4
W014	65.3	6.54	5.00	15.4	0.448	89.6	30.8	34.4	12.9	195	64.7	22.8	9.3	0.61	1.8	1.1
W015	28.9	8.01	5.32	17.2	0.499	93.8	32.3	34.5	13.7	164	49.5	32.3	8.1	0.65	9.2	0.5
W016	21.8	4.90	4.77	14.9	0.448	93.9	31.2	33.3	14.1	190	41.0	44.9	8.8	0.43	3.7	1.4
W017	26.3	8.92	5.77	17.1	0.530	91.9	29.6	32.2	13.4	211	71.1	20.2	6.6	0.59	1.5	0.3
W018	83.9	6.50	4.51	14.6	0.455	100.9	32.4	32.1	12.4	218	68.6	20.3	7.8	0.51	2.0	1.1
W019	16.8	6.22	4.43	13.8	0.407	91.9	31.2	33.9	13.5	182	61.7	24.3	7.2	0.45	5.9	0.6
W020	19.2	6.23	5.35	15.9	0.481	89.9	29.7	33.1	13.0	228	43.5	42.1	9.6	0.60	2.9	1.6
W021	21.3	11.18	3.22	10.7	0.330	102.5	33.2	32.4	13.7	278	59.5	30.3	7.6	0.85	1.2	0.7
W022	17.1	13.12	2.32	7.3	0.228	98.3	31.5	32.0	12.9	511	58.3	32.2	7.0	0.92	1.8	0.5
X001	29.0	13.80	5.09	15.8	0.493	96.9	31.0	32.0	15.0	335	81.2	12.1	4.6	0.63	1.6	0.5
X002	27.9	4.40	4.75	14.5	0.443	93.2	30.5	32.7	14.7	183	72.9	16.4	5.2	0.23	2.3	0.8
X003	21.2	4.92	4.64	14.6	0.467	100.5	31.4	31.3	13.4	150	53.5	34.7	5.7	0.28	2.2	1.3
X004	29.5	6.48	5.58	16.4	0.536	96.0	29.3	30.5	14.2	194	63.4	26.9	5.8	0.38	0.6	0.7
X005	NA	5.00	4.94	14.4	0.447	90.5	29.3	32.3	14.6	266	62.8	25.3	6.0	0.30	2.2	1.1
X006	16.3	8.74	4.73	14.5	0.452	95.6	30.7	32.1	14.0	201	46.6	38.9	11.2	0.98	2.7	0.5
X007	18.4	5.24	4.76	14.7	0.447	93.9	30.9	32.9	13.3	165	45.3	42.4	9.2	0.48	2.5	0.4
X008	10.3	8.36	5.06	14.9	0.455	89.9	29.4	32.7	13.7	230	73.3	17.3	6.7	0.56	1.7	0.6
X009	17.1	4.86	4.99	14.8	0.462	92.6	29.7	32.0	13.4	270	49.2	35.6	8.2	0.40	5.6	1.4
X010	12.3	4.55	4.62	14.2	0.417	90.3	30.7	34.1	13.9	125	76.2	11.0	8.4	0.38	2.6	1.1
X011	17.0	4.98	4.09	13.5	0.398	97.3	33.0	33.9	13.7	243	65.3	23.3	8.6	0.43	1.6	0.8
X012	12.2	4.58	4.96	14.6	0.438	88.3	29.4	33.3	15.2	274	61.2	25.5	9.6	0.44	2.4	1.1
X013	19.6	6.52	5.38	15.7	0.502	93.3	29.2	31.3	15.5	208	60.4	26.2	9.0	0.59	3.4	0.8
X014	18.2	5.40	5.70	17.7	0.546	95.8	31.1	32.4	13.6	203	54.9	30.0	10.0	0.54	4.1	0.6
X015	14.8	8.89	4.92	15.1	0.440	89.4	30.7	34.3	13.7	233	46.2	39.9	10.1	0.90	2.8	0.7

Table A1 continuation

X016	19.1	4.82	5.36	16.4	0.499	93.1	30.6	32.9	13.0	230	53.5	32.0	11.6	0.56	1.7	1
X017	NA	7.14	5.49	13.1	0.469	85.4	23.9	27.9	20.0	277	66.8	21.6	8.5	0.61	2.1	0.7
X018	23.1	6.26	4.74	15.0	0.465	98.1	31.6	32.3	12.2	192	62.9	23.6	9.1	0.57	3.0	1.1
X019	30.6	6.05	4.32	15.1	0.457	105.8	35.0	33.0	14.1	149	66.9	21.5	7.1	0.43	3.5	0.8
X020	NA	8.40	5.32	16.0	0.517	97.2	30.1	30.9	13.8	245	63.6	26.8	8.1	0.68	0.7	0.4
Y001	20.4	6.85	5.03	15.9	0.493	97.9	31.6	32.3	15.5	256	51.6	34.2	9.9	0.68	3.6	0.7
Y002	17.3	6.01	4.87	14.7	0.441	90.6	30.2	33.3	14.1	218	68.0	22.0	5.6	0.34	2.2	0.4
Y003	14.5	3.66	4.21	12.6	0.420	99.8	29.9	30.0	14.3	195	62.9	19.9	11.1	0.41	2.9	1.3
Y004	19.1	5.81	4.84	14.1	0.454	93.8	29.1	31.0	13.8	255	70.8	17.7	7.0	0.41	1.5	0.9
Y005	16.9	5.87	4.28	11.8	0.389	90.9	27.5	30.2	20.6	158	47.7	41.8	6.0	0.38	1.5	0.6
Y006	17.0	7.99	3.79	11.5	0.359	94.7	30.3	32.0	15.6	185	52.8	33.7	10.3	0.82	2.3	0.6
Y007	17.6	6.20	4.77	13.8	0.431	90.4	28.9	32.0	12.7	92	77.1	12.3	8.2	0.51	1.5	0.6
Y008	13.2	5.03	5.09	14.7	0.451	88.6	28.9	32.6	13.3	271	57.3	29.6	9.5	0.48	2.2	0.6
Y009	16.1	5.99	5.10	15.6	0.482	94.5	30.6	32.4	14.7	259	54.4	33.6	8.5	0.51	2.5	0.7
Y010	8.7	8.31	4.32	13.0	0.391	90.5	30.1	33.2	14.6	336	70.3	19.5	6.3	0.52	2.9	0.8
Y011	NA	6.46	4.81	14.7	0.444	92.3	30.6	33.1	13.2	297	48.3	38.2	9.8	0.63	2.9	0.5
Y012	15.9	7.27	4.32	13.3	0.395	91.4	30.8	33.7	12.1	292	47.3	39.3	10.3	0.75	2.2	0.6
Y013	57.4	5.83	4.80	14.6	0.473	98.5	30.4	30.9	14.1	225	56.8	33.4	6.2	0.36	2.1	1.2
Y015	31.1	5.91	4.89	15.0	0.439	89.8	30.7	34.2	12.8	251	54.2	34.5	9.1	0.54	1.5	0.5
Y016	17.9	5.00	4.58	14.2	0.413	90.2	31.0	34.4	13.3	259	70.8	18.0	8.4	0.42	1.6	0.8
Y017	NA	9.13	4.54	13.9	0.448	98.7	30.6	31.0	13.2	305	61.2	24.8	10.2	0.93	2.8	0.7
Y018	20.2	4.02	2.04	9.7	0.300	147.1	47.5	32.3	13.4	609	60.1	27.1	10.9	0.44	0.7	0.7
Y019	22.1	5.93	4.33	13.5	0.452	104.4	31.2	29.9	14.6	356	50.6	35.6	8.8	0.52	3.9	0.8
Y020	27.0	5.82	4.93	15.2	0.468	94.9	30.8	32.5	13.3	259	53.9	34.9	8.8	0.51	1.4	0.7

Key: Fe, Serum iron concentration ($\mu\text{mol/l}$); WBC, white blood cell count ($\times 10^9/\text{l}$); RBC, red blood cell count ($\times 10^9/\text{l}$); Hb., haemoglobin concentration (g/dl); Htct., haematocrit (l/l); MCV, mean corpuscular volume (fl); MCHb, mean corpuscular haemoglobin (pg); MCHbC, mean corpuscular haemoglobin concentration (g/dl); RBCdw, red blood cell distribution width (%); Plat., platelet count ($\times 10^9/\text{l}$); Neut., neutrophil count ($\times 10^9/\text{l}$); Lymph., lymphocyte count ($\times 10^9/\text{l}$); Mon., monocyte differential count (%); Mon.ns, monocyte total count ($\times 10^9/\text{l}$); Eos., Eosinophil count ($\times 10^9/\text{l}$); Bas., Basophil count ($\times 10^9/\text{l}$)

Table A2: APOE genotype of all study participants

Sample ID	Genotype	Sample ID	Genotype
V001	E3/E3	X001	E3/E4
V002	E2/E3	X002	E3/E4
V003	E3/E4	X003	E3/E4
V004	E3/E3	X004	E3/E3
V005	E3/E4	X005	E4/E4
V006	E3/E3	X006	E3/E3
V007	E3/E4	X007	E2/E3
V008	E3/E3	X008	E3/E4
V009	E2/E3	X009	E3/E4
V010	E3/E3	X010	E4/E4
V011	E3/E3	X011	E3/E4
V012	E3/E3	X012	E3/E3
V013	E3/E3	X013	E3/E3
V014	E3/E3	X014	E4/E4
V015	E3/E3	X015	E4/E4
V016	E2/E3	X016	E4/E4
V017	E3/E4	X017	E3/E3
V018	E3/E4	X018	E3/E3
V019	E3/E3	X019	E2/E3
V020	E3/E4	X020	E3/E3
W001	E2/E3	Y001	E2/E3
W002	E4/E4	Y002	E3/E4
W003	E2/E3	Y003	E2/E3
W004	E3/E3	Y004	E3/E4
W005	E3/E3	Y005	E3/E3
W006	E3/E4	Y006	E3/E3
W009	E3/E4	Y007	E3/E4
W010	E3/E4	Y008	E3/E3
W011	E3/E3	Y009	E3/E3
W012	E3/E3	Y010	E3/E4
W013	E3/E3	Y011	E3/E3
W014	E3/E3	Y012	E2/E4
W015	E2/E4	Y013	E3/E3
W016	E2/E3	Y015	E3/E3
W017	E3/E3	Y016	E3/E3
W018	E4/E4	Y017	E2/E3
W019	E3/E3	Y018	E3/E4
W020	E2/E3	Y019	E3/E4
W021	E3/E3	Y020	E4/E4
W022	E3/E3		

Table A3: 16S rRNA gene estimated copy numbers from qPCR analysis

Sample ID	Copy number	Sample ID	Copy number
V001	28.03	X001	86.09
V002	23.45	X002	36.01
V003	29.10	X003	29.64
V004	24.33	X004	20.28
V005	17.93	X005	20.58
V006	20.34	X006	22.94
V007	23.30	X007	23.59
V008	15.91	X008	16.18
V009	34.53	X009	14.85
V010	17.24	X010	39.92
V011	25.42	X011	38.01
V012	22.60	X012	13.04
V013	24.16	X013	23.83
V014	15.00	X014	13.31
V015	14.02	X015	15.28
V016	15.55	X016	10.75
V017	15.94	X017	14.71
V018	26.29	X018	80.48
V019	22.11	X019	18.64
V020	16.00	X020	14.63
W001	33.84	Y001	94.22
W002	28.24	Y002	38.44
W003	24.74	Y003	102.67
W004	14.43	Y004	19.86
W005	23.32	Y005	12.32
W006	16.36	Y006	22.20
W009	12.73	Y007	25.37
W010	12.54	Y008	19.57
W011	17.01	Y009	24.38
W012	11.28	Y010	20.47
W013	79.77	Y011	28.29
W014	27.74	Y012	25.42
W015	17.79	Y013	20.70
W016	27.47	Y015	22.45
W017	12.59	Y016	26.28
W018	26.91	Y017	45.46
W019	29.75	Y018	26.81
W020	34.40	Y019	20.66
W021	29.38	Y020	380.72
W022	85.28		